Metabolism and mode of action of some aromatic amines in relation to carcinogenesis.

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THE METABOLISM AND MODE OF ACTION OF SOME AROMATIC AMINES IN RELATION TO CARCINOGENESIS

"Das Wenige verschwindet leicht dem Blick,

der vorwärts sieht, wie viel noch übrig bleibt".

J.W.von Goethe (1749-1832)

Iphigenie auf Tauris
Act I. Scene II.

ABSTRACT

The clinical, epidemiological and experimental observations leading to the recognition of aromatic amines as carcinogens in man and experimental animals have been recorded.

The metabolism of aromatic amines has been reviewed and current theories on the metabolic processes involved in the activation of aromatic amines to "proximate" carcinogens have been discussed and attention drawn to anomalies in both theories.

The in vitro oxidative metabolism of 4-aminobiphenyl and 4-acetamido biphenyl has been examined and a H . P . L . C . method has been described for the determination of their hydroxylated metabolites. The in vitro deacetylation of some carcinogenic and non-carcinogenic acetamido compounds has been studied using a gas chromatographic technique in several species. The acetylation of aromatic amines with various hepatic systems has been studied.

The synthesis and biosynthesis of 4-aminobiphenylyl-3-glucosiduronate have been investigated and species differences in this metabolic reaction have been recorded.

Examination for carcinogenic activity of 4-aminobiphenyl and three hydroxylated derivatives has been carried out using the mouse neonate technique.

An examination of lysosomal damage caused by a series of aromatic amines and some of their hydroxylated derivatives has been carried out using a lysosomal preparation obtained from dog urinary bladder epithelial cells. The

in vitro activation of a solubilised β-glucuronidase preparation by certain aromatic amines has been observed.

The results obtained are discussed in relation to current concepts of the metabolism of aromatic amines and the role of these metabolites in carcinogenesis.

TO DOREEN AND MY PARENTS

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SECTION 1 INTRODUCTION

1.1 Recognition of Aromatic Amines as Industrial Carcinogens in the Chemical Industry

Whilst ancient civilizations were aware of certain health hazards associated with plant materials which could be used as systemic poisons, there seems to be no early record of the recognition of diseases specifically associated with industry. This is despite the large scale mining of gold by the Egyptians and the widespread use of lead by the Romans.

It was not until the establishment of silver mines in the Harz mountains in 965 A.D., and the subsequent development of the industry over the following six hundred years, that a really comprehensive study of an industry was written. In "De Re Metallica" Agricola in 1556 included a chapter on the health of workers and clearly associated the mining industry with diseases of joints, eyes and lungs occurring in the artisans. Eleven years after the treatise by Agricola, a monograph, specifically devoted to diseases associated with miners and smelters, was written by Paracelsus. This work, "Von der Bersucht und anderen Bergkrankheiten" clearly recognises the poisonous effects of various metals, and differentiates between acute and chronic effects.

The publication "De Morbis Artificum" by Bernardino Ramazzini in 1700 recognised that many other industries were implicated in the ill-health of workers. This classic work has had a tremendous influence on the recognition of industry as a potential hazard to health, and Hunter (1959) rightly describes Ramazzini as the Father of Occupational Medicine.

From these initial observations the discipline of Industrial Health has developed; obviously the advent of the Industrial Revolution resulted in more and more people becoming exposed to industrial situations where diseases could be induced.

The first description of cancer occurring as an industrial disease is attributed to Percival Pott (1775), who observed a high incidence of scrotal tumours in chimney sweeps, and suggested that this was due to intimate contact with soot. As industry developed it was soon realised that many other materials, to which workers were exposed, were able to induce skin tumours. Volkmann (1875) observed skin tumours in workers exposed to paraffin, and Bell (1876) made a similar observation among workers in the shale oil industry in Scotland.

The implication of aromatic amines as industrial carcinogenic hazards began with the observation by Rehn (1895), who reported the occurrence of cancer of the urinary bladder in three men employed in the manufacture of magenta. Rehn suggested that aniline, an aromatic amine used as an intermediate in the synthesis of magenta, was the causative agent. The German chemical industry, prior to the First World War (1914-1918), was paramount. Rehn's observation was soon followed by others, so that by 1942 over three hundred cases of cancer of the urinary bladder had been found in workers in the German chemical industry and attributable to exposure to aromatic amines (Heuper, 1942).

With the development of a chemical industry in other countries, either as a result of economic development or war, cancer of the urinary bladder became an international problem. In Switzerland tumours were observed in chemical workers by Schedler (1905) and in Great Britain the first cases were reported by Ross (1918). Henry, Kennaway and Kennaway (1931) observed that during the period 1921 to 1928 sixty-one fatalities due to cancer of the urinary bladder occurred in chemical workers in Great Britain.

Heuper (1942) has documented a further thirty cases reported up to . 1937.

In other countries a similar situation exists. In Austria, Scheele (1926) and Schueller (1932) reported the occurrence of bladder tumours in chemical workers, and in the Soviet Union bladder tumours have been reported in chemical workers by Rosenbaum and Gottlieb (1926) and Abramyan, Rothberg and Mayants (1933).

In the United States of America, Ferguson, Gehrmann and Gay (1934) reported twenty five cases all originating from one chemical concern, and by 1942 Heuper estimated that about one hundred cases had been detected in thatcountry. In 1937 di Maio reported twelve bladder tumours occurring amongst eighty-six workers in one Italian dye factory. Nagayo and Kinosita (1940) described three urinary bladder papillomas arising in workers in the Japanese chemical industry.

With regard to the nature of the responsible agent in the etiology of this industrial disease, little support can be found for the suggestion by Rehn (1895) that aniline may be involved. Walpole, Williams and Roberts (1952) suggested that bladder tumours were caused by naphthylamines and xenylamines occurring as impurities in the manufacture of dyestuffs. This idea received further support following the publication of an epidemiological survey of workers in the British chemical industry. In this report Case, Hosker, McDonald and Pearson (1954), Case and Pearson (1954), indicated that exposure to aniline was not responsible for the bladder tumours found in workers in the chemical industry, but that 1- or 2-naphthylamine,

benzidine (4,4'-diaminobiphenyl) or magenta presented a very real risk to the workers engaged in their manufacture.

Another potent industrial carcinogen, with an action on the urinary bladder, was recognised by Melick, Escue, Naryka, Mezera and Wheeler (1955) who reported that 11% of workers exposed to xenylamine (4-amino-biphenyl) developed bladder tumours. These workers have been subjected to extensive "follow up" and cytological studies (Koss, Melamed and Kelly, 1965; Koss, Melamed, Ricci, Melick and Kelly, 1969; Melick, Naryka and Kelly, 1971) and some of their results are shown in Table 1.1. These publications also indicate the value of exfoliative cytology in the detection of the disease, and Melamed (1972) has clearly shown that bladder cancer can develop even though exposure to the carcinogenic stimulus had ceased some years before the tumour was clinically recognised. During the fourteen years following the cessation of production of 4-aminobiphenyl, eighty-six workers developed a suspicious or positive cytology, and of these, forty-three subsequently had histologically confirmed cancer of the urinary bladder.

Studies were also carried out on workers in the Italian chemical Industry where benzidine was still being produced (Maltoni and Ghetti, 1964) and a number of cases of carcinoma of the bladder were detected.

Follow up studies by Forni, Ghetti and Armeli (1972) again indicated the value of cytological studies of urinary deposits because,of the sixteen new cases found, fourteen had been predicted by the technique described by Crabbe (1952).

Table 1.1.1 Incidence of cancer of the urinary bladder found in men employed in two plants and exposed to 4-aminobiphenyl

(from Melick et al. 1971)

Plant	Year	Men examined	Men with bladder tumours	%
1	1953	71	12	16.9
	1958	186	23	12.4
	1970	261	42	16.1
2	1953	44	1	2.3
	1958	45	2	4.4
	1970	54	10	18.5

In recent reports the hazard from exposure to benzidine has again been emphasised. Zavon, Hoegg and Bingham (1973) found that of twenty-five men exposed to benzidine, thirteen had clinically defined cancer of the bladder and Wendel, Hoeg and Zavon (1974) reported on the relatively short induction time of the disease with this carcinogen in man.

1.2 Exposure to Aromatic Amines as a Carcinogenic Risk in other Industries or Environments

The evidence presented in the preceding section clearly implicates certain aromatic amines as causative factors in the etiology of bladder cancer observed amongst workers exposed to them in the chemical and dyestuff industry. Indeed the bulk of aromatic amines were originally specifically synthesised for use in the dyestuff industry as dyestuff intermediates, and generally they were transformed into harmless dyes (Case, 1966a).

In addition, small but significant amounts were purified and sold as fine chemicals for laboratory use. Later there developed a requirement for aromatic amines in other industries either as the parent amines or as specialised products.

During the course of the survey carried out by Case and his colleagues (1954), a fortuitous observation was made in the general population, then being used as a control. An unexpected number of patients who suffered from bladder tumours had been employed in one large rubber works in Birmingham. Case and Hosker (1954) calculated that during the period 1936-1950 only four cases of cancer of the urinary bladder would have been expected amongst all the skilled rubber-workers in Birmingham, if no industrial risk were involved; in practice twenty-two cases were discovered. Subsequent studies (Case, 1966b) continued to show that workers in the British rubber industry are "at risk".

It was soon realised that the causative agents in the disease were probably the antioxidants incorporated into the rubber to prevent perishing. These

antioxidants were produced from 1-and 2-naphthylamine by condensation with acetaldehyde, and contained 2.5% free naphthylamines of which 0.25% was 2-naphthylamine. These antioxidants were sold under the trade name "Nonox S" and "Nonox HF". Another antioxidant "Santoflex BX" contained 4-aminobiphenyl (Anon. 1966a).

A similar situation has been observed in the United States of America, where Guira (1971) examined the records of bladder cancer patients at four hospitals in Akron, Ohio. Of the 585 patients with bladder cancer, Guira found that 168 were employed in the rubber industry and 90% of these were employed by only three companies. Cole, Hoover and Friedell (1972), in a survey of workers in Eastern Massachusetts, found a high incidence of bladder cancer amongst workers employed in the dyestuff, organic chemicals, rubber, paint and leather industries. Workers in these latter two industries could well be exposed to amines occurring as impurities in the pigments and dyes used.

Perhaps not surprisingly another industry where rubber was extensively used soon became suspect as a source of industrial bladder tumours. In 1965 Case and Davies reported on the high incidence of bladder tumours in workers in the electric cable industry which was apparently due to their exposure to aromatic amines occurring as impurities in the condensed anti-oxidant (Davies, 1965).

As early as 1931 Henry, Kennaway and Kennaway indicated that workers in retort houses producing gas developed bladder tumours. This finding was substantiated in further surveys by Doll et al. (1965, 1972) who draw attention to the finding by Battye (1966) of 2-naphthylamine in tar fumes and condensate.

The compound α-naphthylthiourea (ANTU) was introduced into our environment as a potent rodenticide. The commercial compound contains approximately 1% free naphthylamines of which about one-fifth is the 2-isomer (Anon. 1966b). Two occurrences of bladder cancer were reported in users of ANTU (Anon, 1966c). Veys (1969)described a further case reported to H.M.Coroners Notification Scheme.

Aromatic amines are used in laboratories for many purposes; most chemists are exposed to them during their training due to their use as 'spots' in order to gain expertise in chemical analysis or organic synthesis. Benzidine was routinely used in pathology laboratories for detection of blood in stools (Lehmann, 1967), and as a spray reagent for detection of sugars on paper chromatograms. Searle (1970) has drawn attention to the possible risk to laboratory workers of a whole variety of organic chemicals and The Department of Education (1970) issued a pamphlet on the use of aromatic amines in schools and educational establishments. The only survey of chemists was carried out amongst members of The American Chemical Society by Li, Fraumeni, Mantel and Miller (1969). No higher incidence of cancer of the urinary tract was observed amongst this profession compared with a comparable group of professional men in general.

Veys (1969) has indicated that workers in the textile and printing industries may also be "at risk" due to their exposure to dyestuffs which may contain aromatic amines or their derivatives. It should also be remembered that benzidine has been used to impregnate paper and is present in printing

materials for the manufacture of security papers.

In addition to cancer of the pulmonary system cigarette smoking has been alleged to be a causal factor in a large number of other diseases. A number of reports indicate that cigarette smoking is associated with cancer of the urinary bladder (see Gorrod, Jenner, Keysell and Mikhael, 1974, and references cited therein,and Armstrong and Doll, 1974). Small amounts of naphthylamines and aminofluorene have been detected in cigarette smoke by Pailer, Hubsch, and Kühn (1967). More recently Masuda and Hoffmann (1969) have described quantitative methods for determination of 1-and 2-naphthylamine occurring in cigarette smoke. These authors reported a total of fifty nanograms of naphthylamines occurring in the smoke of one cigarette, about forty percent of which was the 2-isomer.

Naphthylamines could have been formed via reaction of ammonia with naphthalene or naphthol in the heated zone of the cigarette or by the pyrolysis of L-glutamic acid or L-leucine as described by Masuda, Mori and Kuratsune (1967).

It is of interest that Wagle & Lee (1973) have recently claimed to induce bladder tumours by direct application of tobacco tar to the urinary bladders of rats.

The majority of drugs used in clinical practice are nitrogenous compounds, although only few of them having the nitrogen atom directly attached to an aromatic system. At present two drugs, which could be converted into aromatic amines in vivo, have been demonstrated to be

bladder carcinogens in humans.

The first indication that chlornaphazin (N,N-bis-(2-chloroethyl)-2-naphthylamine) was carcinogenic to man, came in the report by Chievitz and Thiede (1962). These authors had used the drug in Denmark for the treatment of Hodgkin's disease and polycythaemiavera, and later found that three cases of cancer of the bladder occurred amongst twenty-six patients treated with this drug. Later, Videback (1964) reported a further nine cases which he attributed to the treatment of patients with chlornaphazin.

Boyland & Manson (1963) have shown that chlornaphazin is converted in rats to metabolites which could have been derived from 2-naphthylamine, following initial dealkylation.

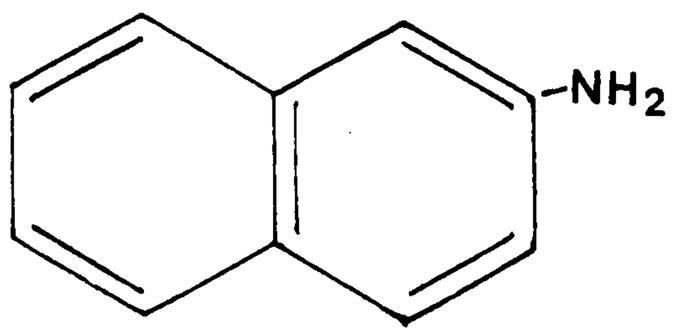
The second drug which is clearly implicated in toxicity towards the human urothelial system is phenacetin. This drug was introduced as an analgesic-antipyretic in 1866, and throughout the ensuing years it has gained in popularity and, until recently, was widely used throughout the world. The toxicity associated with phenacetin and some related derivatives of p-aminophenol has recently been reviewed by Godfrey (1972) and Shelley (1967). The easy availability of this drug has lead to abuse in certain individuals. In many cases the individual first required the analgesic due to his employment. Thus watch-makers and others doing close work and workers in ordinance factories exposed to nitro-compounds, often take analgesics daily to treat headaches.

Usually, phenacetin abuse is associated with nephrotoxicity leading to transitional-cell tumours of the renal pelvis (Bengtsson and Angervall, 1970; Begley, 1970), although tumours of the urinary bladder occur with a lower frequency (Bengtsson, Angervall, Ekman and Lehmann, 1968; Begley, Chadwick

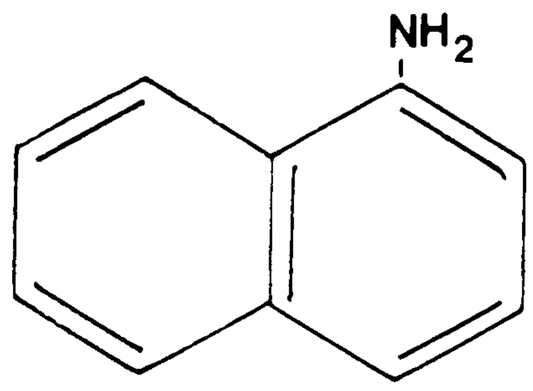
and Jepson, 1970). More recently Manion and Susmano (1971) have described a further bladder tumour which they ascribe to phenacetin abuse. In all the cases of bladder cancer attributable to phenacetin, it should be noted that the individuals each had a yearly consumption in excess of one kg of the drug.

Phenacetin is deacetylated in man to yield an aromatic amine, p-phenetidine, although this appears to be a minor pathway of metabolism (Brodie and Axelrod, 1949). It may be that further metabolism of p-phenetidine occurs, as described by Büch, Hauser, Pfleger and Rudiger (1966), and the true significance of this deacetylation route is more important than hitherto thought.

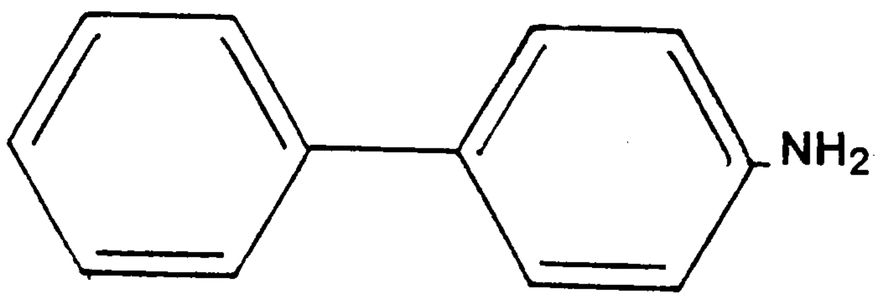
The structures of compounds discussed in Sections 1.1 and 1.2 are shown in Fig.1.2.1.



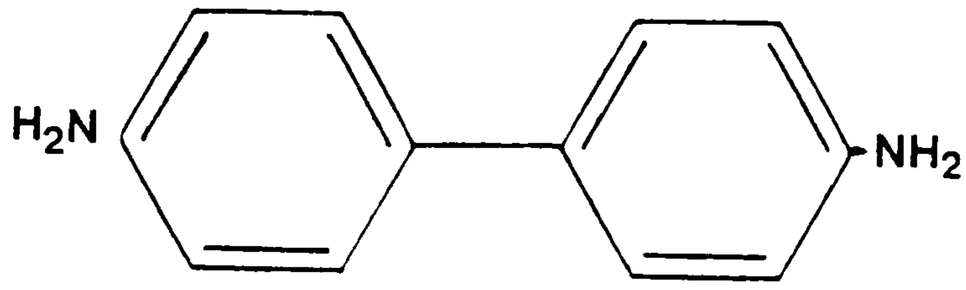
2-naphthylamine



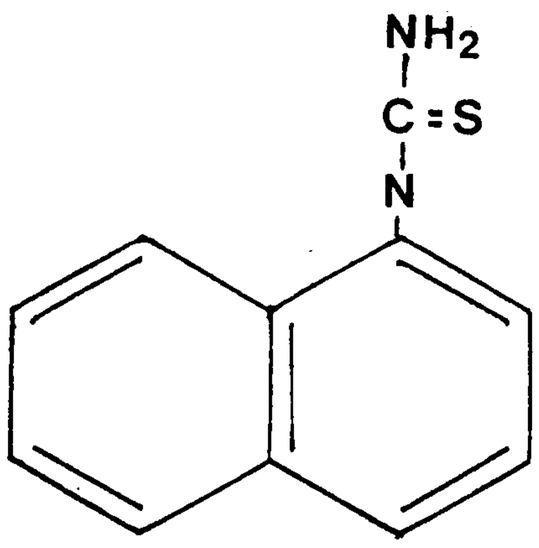
1-naphthylamine



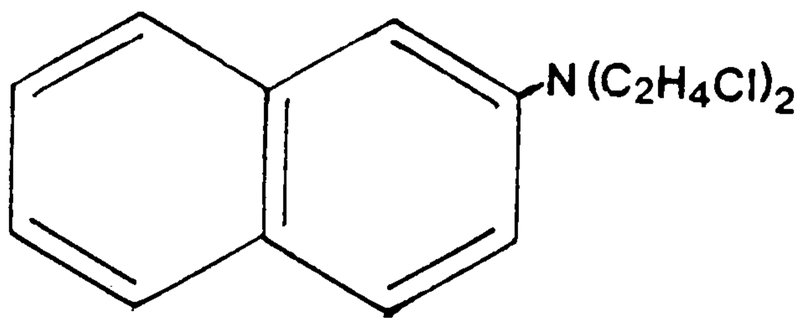
4-aminobiphenyl
(p-xenylamine)



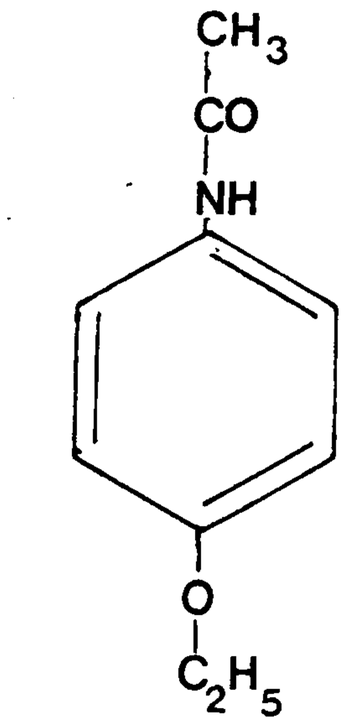
4,4'- aminobiphenyl
(Benzidine)



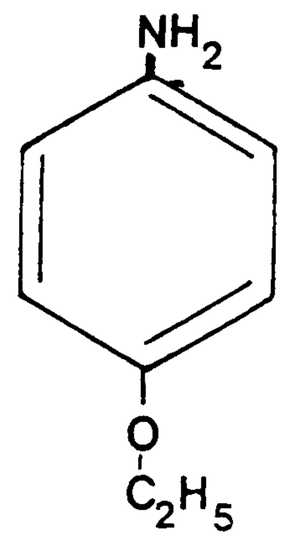
N-1- naphthylthiourea
(ANTU)



N, N-bis(2-chloroethyl)-2-
naphthylamine (chlornaphazin)



phenacetin



phenetidine

Fig.1.2.1 Structures of some aromatic amines and derivatives discussed in Sections 1.1 and 1.2.

1.3. Production of Cancer by Aromatic Amines in Experimental Animals

The production of neoplastic disease is unlike many other toxic phenomena in that, usually, a considerable time elapses from the first exposure to the chemical until the first appearance of a clinically recognisable tumour. This latent period varies very much with the animal species studied. In man, Case et al. (1954) reported a mean latent period of seventeen years for workers exposed to benzidine and naphthylamines. A similar figure is apparent from the work of Wendel et al. (1974) for workers involved in benzidine manufacture. Case and Pearson (1954) reported a similar latent period for workers exposed to auramine and magenta.

The requirement to produce the disease in experimental animals was initiated with two specific aims in mind. Firstly there was the need to confirm which substances in our environment were really able to cause bladder cancer and which substances were ineffective. The second reason for reproducing the disease in experimental animals was the need to have a model system in which the initiation, development and the treatment of the disease could be studied.

Animal species vary in their ability to utilise any specific metabolic pathway for foreign compounds (See Section 1.4); they also vary in their sensitivity to a carcinogenic stimulus and in the latent period for any specific carcinogen. Therefore, only when the correct combination of species, together with the appropriate dosage schedule is used is it possible to produce the disease in experimental animals.

The first reports of the production of tumours of the urinary bladder in rabbits, following subcutaneous administration of 2-naphthylamine (Schar 1930; and Perlmann and Stachler, 1932, 1933) have since been discredited

by Bonser (1943) and Heuper, Briggs and Wolfe (1938). In fact Berenblum and Bonser (1937) administered various amino compounds over long periods to tabbits and rats by injection, feeding or inhalation, but failed to produce tumours of the urinary bladder or other organs. In the same year Heuper and Wolfe (1937) reported, in a preliminary communication, on the successful production of bladder neoplasms by administering commercial 2-naphthylamine subcutaneously to female Beagle dogs. A full report of their experiments was published in 1938 (Heuper, Wiley and Wolfe) which showed that 2-naphthylamine was able to produce a whole spectrum of urinary bladder lesions ranging in severity from hyperplasia to carcinoma. These results were confirmed by Bonser (1943) who administered pure 2-naphthylamine orally to mongrel dogs. In contrast, oral administration of 1-naphthylamine to dogs by Bonser, Clayson & Jull (1958) indicated that this compound was virtually inactive.

Conzelman and Moulton (1972) also administered 2-naphthylamine to Beagle dogs and found that the total amount of this compound required to produce bladder tumours was much less when small daily doses of 6.25 mg/Kg were given, compared to the animals receiving 25 or 50 mg/kg. This is in contrast to the findings of Saffiotti, Cefis, Montesano and Sellakumar (1967) who found that only high doses of 2-naphthylamine produced bladder cancer, after oral administration to Syrian hamsters. Bladder cancer was also produced by 2-naphthylamine when given to Rhesus Monkeys (Macaca mulatta) by Conzelman, Moulton, Flanders, Springer and Crout (1969). It is of interest that of all the other adult animals treated with 2-naphthylamine, i.e. rats, mice, guinea pigs and rabbits only in mice was there a significant carcinogenic

response. In this species, Bonser, Clayson, Jull and Pyrah (1952) reported the formation of hepatomas.

4-Aminobiphenyl was reported by Walpole, Williams and Roberts (1954) to be carcinogenic to the bladder when fed to dogs; this result was confirmed by Deichmann et al. (1956;1965a; 1965b).). The rabbit was also shown to develop bladder cancer after exposure to 4-aminobiphenyl (Bonser, 1962). When this amine was given by stomach tube to female mice, a remarkable strain difference in response was apparent. When Ab x I.F. mice were used the liver was resistant to the formation of hepatomas by this agent, whereas when C57 x I.F. mice were used a high incidence of hepatomas was observed. (Clayson, Lawson & Pringle, 1967). In the former strain a low incidence of bladder tumours was also observed (Clayson, Lawson, Santana and Bonser, 1965). Walpole, Williams and Roberts (1952) showed that 4-aminobiphenyl produced intestinal tumours when given subcutaneously to rats; in this same publication they also showed that the 3,2'-dimethyl derivative was much more potent than the parent amine. This latter compound is of special interest as again it indicates a genetic factor involved in determining the site of tumour formation upon exposure to an aromatic amine. Thus, this compound which produced early intestinal neoplasms in Wistar rats, elicited a high incidence of bladder tumours when administered to Slonaker rats. So and Wynder (1972) have reported a high incidence of bladder tumours in Syrian golden hamsters receiving 3,2'-dimethyl-4-aminobiphenyl subcutaneously. Walpole and Williams (1958) also reported on the carcinogenicity of a variety of methyl derivatives of 4-aminobiphenyl: in nearly every case the

primary lesions seemed to be associated with the intestinal tract. The exception to this was 4-amino-2-methylbiphenyl which did not produce intestinal tumours but produced liver tumours instead. In other carcinogenesis experiments with 4'-halogeno-4-aminobiphenyls, the 4'-fluoro compound was a powerful carcinogen, affecting the kidneys, liver and intestines, whereas the chloro or bromo analogues were inactive even after two years of treatment. (Walpole & Williams, 1958). When the chloro substituent was in the 3-position the ability to produce intestinal neoplasms was retained. These authors also showed that 4-amino-3-methoxybiphenyl was able to produce bladder tumours after subcutaneous administration to rats. This result is very similar to that obtained by Hackmann (1956) who produced bladder tumours in rats after feeding 2-amino-3-methoxy-diphenylene oxide, a finding more recently confirmed by Radomski, Brill and Glass (1967).

Benzidine is another compound implicated in the etiology of the human disease (Section 1.1). This amine has also been tested in experimental animals and has produced bladder carcinoma when given to dogs (Spitz, quoted by Bonser, 1962), although in rats and mice hepatomas were produced (Spitz, Maguigan and Dobriner, 1950; Pliss, 1964). Bonser, Clayson & Jull (1956) were unable to produce bladder tumours in mice with this compound, although hepatomas were again produced in this species. In rabbits only one of twelve animals developed a bladder carcinoma as a result of exposure to benzidine (Bonser, 1959). Benzidine was also shown to produce cholangiomas, hepatomas and liver cell carcinomas (Saffiotti, Cefis, Montesano & Sellakumar, 1967; Sellakumar, Montesano and Saffiotti, 1969).

Deichmann et al. (1965) fed benzidine to pure-bred beagle dogs at a dose of 1.0 mg/kg five times a week for approximately three years without the animals developing bladder tumours. At the same dose level 2-naphthylamine also failed to induce bladder neoplasms, whereas 4-aminobiphenyl was a potent bladder carcinogen at this dose level.

Many other aromatic amines have been tested in experimental animals for their ability to produce neoplasms. In some cases these studies were conducted because of the use or proposed use of the compound either in the dyestuff industry or because of another specific use of the compound. In other cases the amines were tested, because of structural similarity with known carcinogens, in the hope that a rational basis for the prediction of the chronic toxicity of aromatic amines would emerge.

In the former category, 3,3'-dimethylbenzidine (o-tolidine), a compound widely used as a dyestuff intermediate in the manufacture of more than ninety-five dyes, 3,3'-dimethoxybenzidine (o-dianisidine) as intermediate for about ninety dyes, and 3,3'-dichloro benzidine have all been tested. These amines seem to be weaker carcinogens than the mono-amines, producing a rather wider spectrum of tumours, but at a much lower incidence. Surprisingly, with rats, the liver seems resistant to these carcinogens and the predominant site of action is Zymbal's gland (Pliss and Zabezhinsky, 1970). In the hamster, 3,3'-dimethoxybenzidine and 3,3'-dichlorobenzidine caused a low yield of bladder tumours, whilst 3,3'-dimethylbenzidine was inactive (Saffiotti et al. 1970). This is in contrast to the activating effect of an adjacent methyl group seen during carcinogenicity studies on derivatives of 4-aminobiphenyl and 2-naphthylamine. 3-Methyl-2-naphthylamine is a very potent carcinogen in

the rat, even producing tumours at the site of administration (Shenoy, Ambaye & Panse, 1964; Weisburger et al. 1967).

As early as 1938, Shear had shown that 2-anthramine was a very potent hepatoma-producing agent when given to mice, a result confirmed by other workers, whereas Bielschowsky (1946, 1949) showed that both 1- and 9-anthramine were inactive. This specific structural requirement is similar to that observed with naphthylamines and aminobiphenyls, where 1-naphthylamine and 2-aminobiphenyl were virtually inactive compared with 2-naphthylamine and 4-aminobiphenyl.

Work on what must be the most widely studied nitrogen-containing carcinogen started in 1941 when Wilson, De Eds and Cox reported that §2-acetamidofluorene was a carcinogenic substance when fed to rats. This amide, which produced tumours in the liver as well as many other organs, was originally patented as an insecticide. Further studies showed that, with the exception of the guinea pig, every species subjected to this material ultimately developed a tumour. The site and nature of the tumour depended upon the species, route of administration and hormonal status of the animal. So many studies have been carried out with this compound that it is impossible to cite them in detail. In 1958 Weisburger and Weisburger reviewed all aspects of 2-acetamidofluorene carcinogenicity and this was subsequently updated by Arcos and Argus (1968)

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§ The nomenclature for compounds containing the group $ArNHCOCH_3$ varies. They are described as acetamido, acetylamino or N-arylacetamides depending upon the literature source, country of origin and the date of publication. Chemical Abstracts uses the latter system; however this can become clumsy in a descriptive text and the former type of nomenclature has been adopted throughout this thesis.

Unlike the other compounds discussed in this section, 2-acetamidofluorene is an amide and not an aromatic amine. However, amides can be hydrolysed biologically (see Section 3) to produce the corresponding amines and Arcos and Argus (1974) have called these amine-generating compounds Auxocarcinogens. In addition to acetyl groups, other acyl substituents can be enzymically cleaved to produce aromatic amines. Similarly, N-alkylated amines can be metabolised by an oxidative process to yield the corresponding amine. It is not surprising therefore that other amides were soon recognised as carcinogenic substances. These include a wide range of substituted derivatives of 2-acetamidofluorene (for details see Arcos and Argus, 1974), 4-acetamidobiphenyl (Jabra, 1963) and 2-acetamidophenanthrene. This latter amide is rather unusual in that it has the ability to produce leukaemia when given to rats (Miller, Sandin, Miller and Rus ch, 1955).

In the same way as acetamides or other amides may be metabolic precursors of aromatic amines as discussed earlier, it is possible for aromatic nitro-compounds to be converted into aromatic amines by in vivo biological reduction (Mitchard, 1971). It is not surprising therefore to find that some nitro-compounds are carcinogenic when administered to experimental animals. Deichmann et al. (1958) found bladder tumours were produced in dogs following the oral administration of 4-nitrobiphenyl. Similarly, after 2-nitronaphthalene was fed to a monkey multiplepapillomas were found in the urinary bladder by Conzelman, Moulton and Flanders (1970), although frank carcinoma was not seen in this case.

The nitro analogue of benzidine, 4,4'-dinitrobiphenyl has been tested in rats by Laham, Grice & Sinclair (1964). This compound produced ear duct and mammary adenocarcinomas in female rats but was inactive in males.

From the foregoing it can be seen that the original idea that certain aromatic amines were etiological factors in the production of certain human tumours was well founded. Not only were the compounds suspected of being carcinogenic towards man, proven to be carcinogenic when given to experimental animals, but also a range of hitherto unsuspected compounds were shown to be carcinogenic and are therefore recognised as a hazard when allowed into our environment.

In order to produce these tumours in experimental animals, it was generally necessary to expose the animal to multiple dosing for a large part of its life span. Often tumours were only produced when high doses were given and even then some species remained refractory to the stimulus. The many studies in this field show that no clear structure–activity relationship is apparent; usually if one isomer of an amine or amide is active, other isomers will be inactive or very weakly active.

Additionally it has been shown that not only are aromatic amines active in their ability to produce tumours, but also, that compounds which are metabolised to aromatic amines have this property. It is not suggested that conversion to an aromatic amine is a prerequisite for activity in every case, and some compounds may have this property per se or after metabolic conversion via an alternative pathway. This concept will be discussed later in this thesis. The tumour producing activity of some aromatic amines, acetamido compounds and nitro compounds is summarised in Table 1.3.1. This table is not comprehensive and for further details reference must be made to monographs by Clayson (1962), Arcos and Argus (1974), Boyland (1963), Scott (1962) and Temkin (1957).

The structures of some of the compounds discussed in this section are shown in Fig.1.3.1.

In view of the data presented in this section it would be easy to conclude that aromatic amines are so toxic that their use in human therapy is negated. However, this is not the case, and drugs such as p-amino-salicylic acid, the sulphonamides, Dapsone (4,4'-diaminodiphenylsulphone), Procaine, Procainamide and Benzocaine have been widely used in human therapy for many years without being associated with a cancer-producing risk. Another aromatic amine 6-aminochrysene (Chrysenex) has been used both in the treatment of human mammary carcinoma and chronic myeloid leukaemia (Gelzer and Loustalot, 1967; Buu-Hoi, Huong, Lu and Tuong, 1962). This compound was not carcinogenic when administered to adult rats or mice (Lambelin, Mees and Buu-Hoi, 1967).

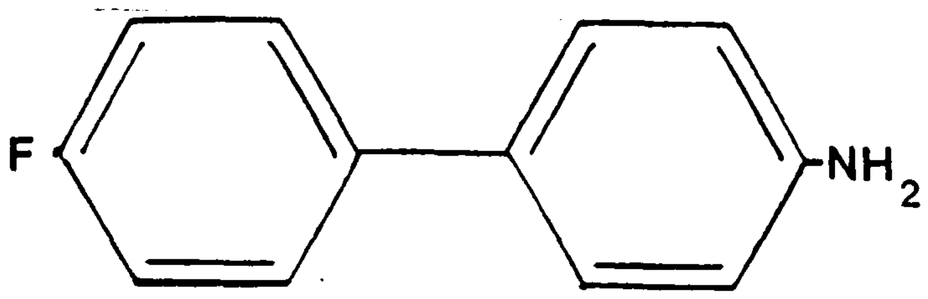
Table 1.3.1 Sites of principal tumours produced by various amines or their metabolic precursors in animal species

Compound	Species	Site of Action
2-Naphthylamine	man	urinary bladder
	dog	urinary bladder
	hamster	urinary bladder
	mouse	liver
	monkey	urinary bladder
4-Aminobiphenyl	man	urinary bladder
	dog	urinary bladder
	rat	intestine
	mouse	liver
	rabbit	urinary bladder
4-Amino-4'-fluorobiphenyl	rat	kidney
Benzidine	man	urinary bladder
	dog	urinary bladder
	mouse	liver
	hamster	liver
4-Amino-3-methylbiphenyl	rat	intestine
4-Amino-3,2'-dimethyl-biphenyl	rat	intestine
	hamster	urinary bladder
4-Amino-3-methoxy-biphenyl	rat	urinary bladder
4-Amino-3-methoxy-biphenylene oxide	rat	urinary bladder
2-Anthramine	mouse	liver

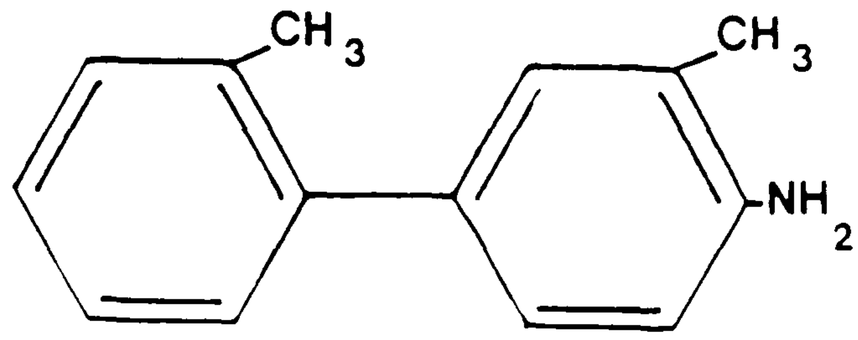
contd..

Table 1.3.1 (continued)

2-acetamidofluorene	rat	liver and mammary gland
	mouse	liver and mammary gland
	hamster	liver
	rabbit	urinary bladder and kidney
	dog	liver and urinary bladder
	cat	lung
2-acetamidophenanthrene	rat	leukaemia
4-acetamidobiphenyl	dog	urinary bladder
4-nitrobiphenyl	dog	urinary bladder
4,4'-dinitrobiphenyl	rat	mammary gland
2-nitronaphthalene	monkey	urinary bladder

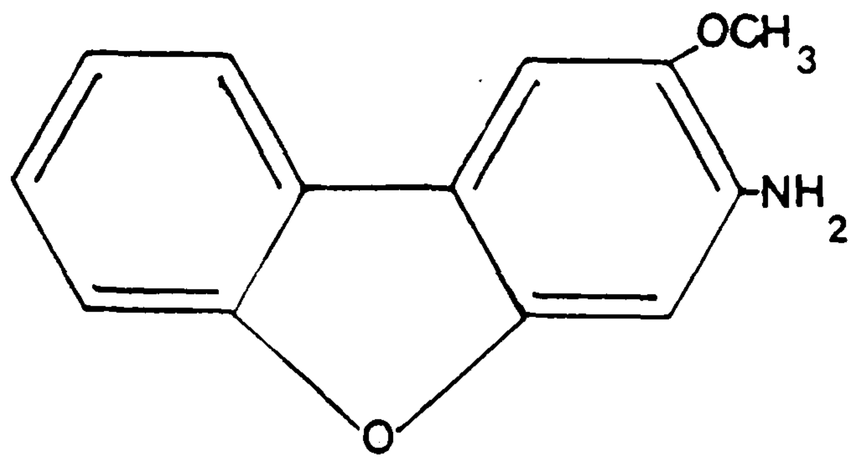


4-amino-4'-fluorobiphenyl

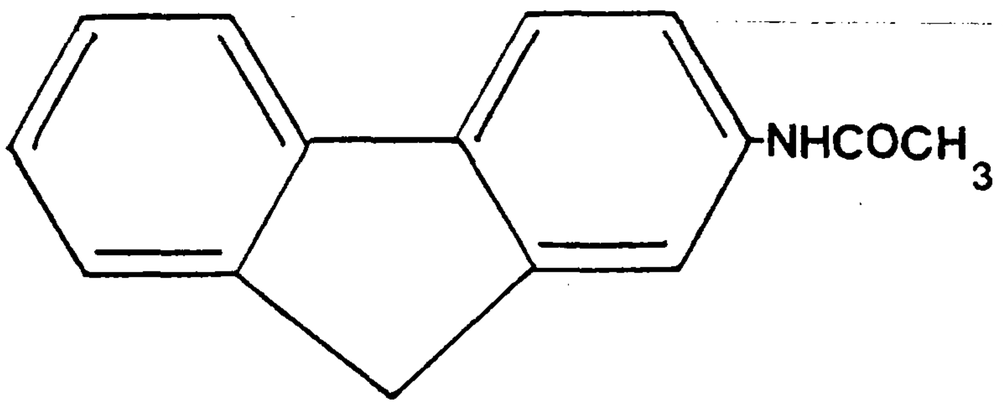


4-amino-2'-3-dimethylbiphenyl

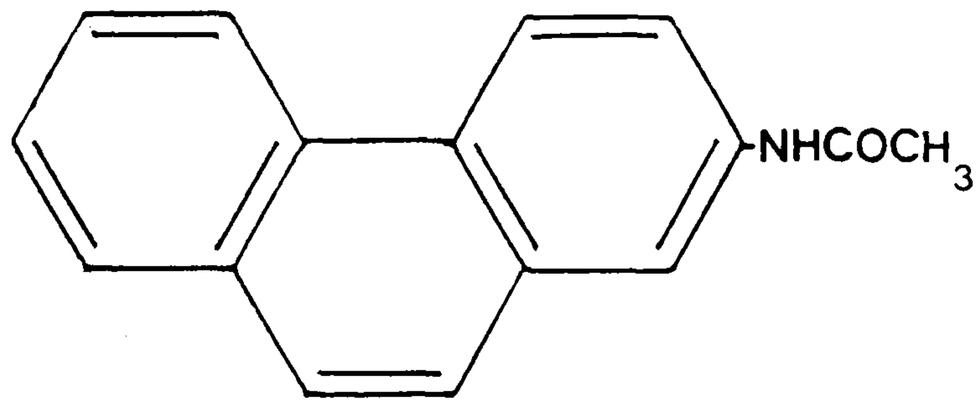
4-amino-3-methoxybiphenyl



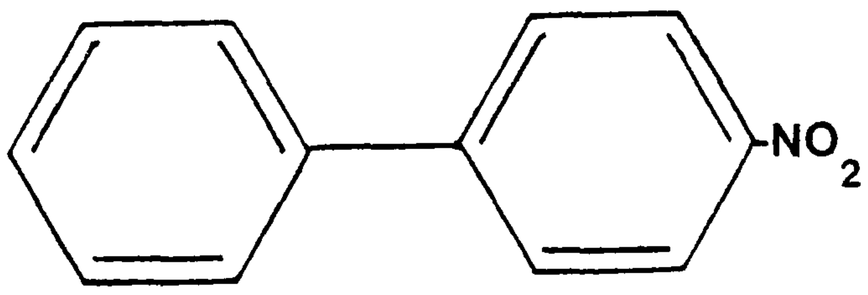
4-amino-3-methoxybiphenyleneoxide



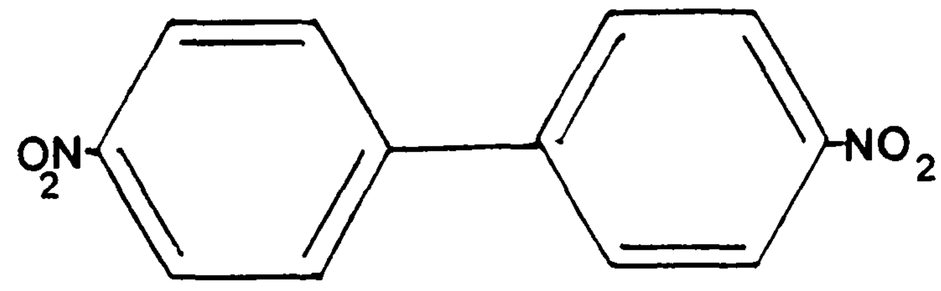
2-acetamidofluorene



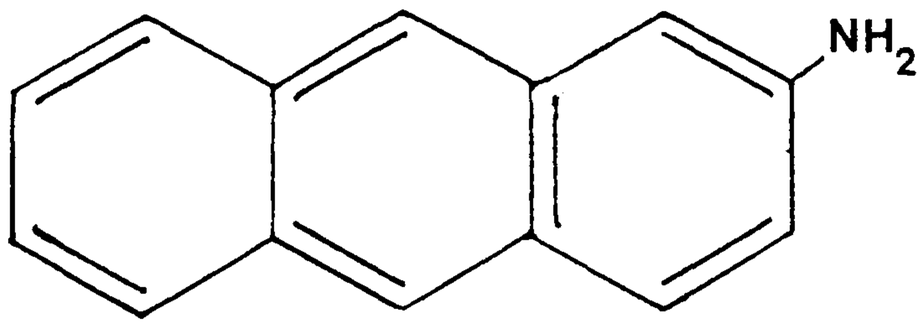
2-acetamidophenanthrene



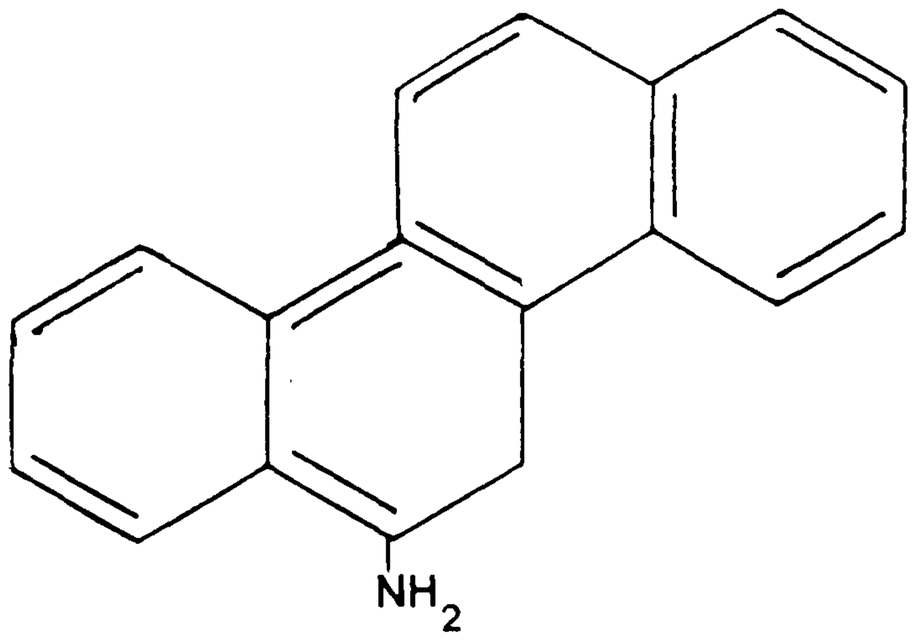
4-nitrobiphenyl



4,4'-dinitrobiphenyl



2-aminoanthracene



6-aminochrysene

Fig.1.3.1. Structures of some compounds discussed in section 1.3.

1.4 Metabolism of Aromatic Amines

When aromatic amines are given to experimental animals, the pharmacological or toxicological response, in the majority of cases, is at a site anatomically distant to that of the site of administration. This suggests that the amine may be acting via a metabolite rather than the parent compound, as otherwise local effects would have been expected. Other explanations, such as tissue susceptibility, tissue distribution or localisation are tenable for certain compounds, but in order to fully account for the toxicological or carcinogenic effects of aromatic amines the metabolism of these compounds appears a prerequisite.

The aromatic amines discussed in the previous sections are composed of four distinct moieties, consisting of the essential amino group, and the aromatic nucleus (Ar). In addition, some aromatic amines have nuclear substituents (R^1) or substituents on the nitrogen atom (R^2 and R^3).(Fig.1.4.1). Each of these groups can be the site of a metabolic reaction. In many cases the initial reaction which is often oxidative, provides a functional group. This is usually followed by a conjugation reaction, thereby converting the molecule into a highly ionised water-soluble compound allowing excretion, via the kidney, into the urinary tract. This two-phase concept of foreign compound metabolism was originally proposed by Williams (1959) and is exemplified by the compound aniline. Aniline has been studied by many workers, originally by Schmiedeberg (1878) and Muller (1887), and more recently by Smith and Williams (1949). Schmiedeberg(1878) recognised that aniline was probably converted to p-aminophenol and then conjugated with sulphuric acid, but it was only with the systematic studies of Smith and Williams (1949) and of Parke (1960) who used ^{14}C-aniline that the true complexity of the metabolic picture emerged.

As aromatic amines already possess a functional group, some metabolic reactions involve the aromatic amino group without a prior oxidative reaction. Thus aromatic amines are converted into N-glucosiduronic acids or N-sulphates (sulphamates) both in vitro and in vivo. The former metabolites were indicated from the work of Smith and Williams (1949a) in studies on the metabolism of aniline, phenetidine and anisidines. These N-glucosiduronates were first isolated by Bushby and Woiwod (1955, 1956) from rabbit urine, following the administration of 4,4'-diaminodiphenylsulphone (Dapsone). Boyland, Manson and Orr (1957) showed that this was also a route of metabolism for 2-naphthylamine. These authors also showed that aniline and 2-naphthylamine could be converted to sulphamic acids. Since then 3,4-dimethylaniline (Boyland & Sims, 1959), benzidine (Clayson, 1959), 4-aminobiphenyl (Bradshaw, 1959), 4-amino-2',3-dimethylbiphenyl and 4-amino-3-methylbiphenyl (Gorrod, 1960), have all been shown to form both N-glucosiduronates and sulphamates.

Boyland, Manson and Orr (1957) showed that aromatic amines react spontaneously with glucuronic acid to form N-glucosiduronates and it may be that some are formed by this mechanism. It seems likely however, that under physiological conditions, these compounds can be synthesised by transfer of the glucuronic acid moiety from uridine diphosphoglucuronic acid (UDPGA) under the influence of a microsomal glucuronyl transferase. This proposal would be in accord with the accepted mechanism for the formation of other glucosiduronates, including phenolic glucosiduronates.

The biosynthesis of arylsulphamates has been studied by Roy (1960) who indicated that, in a similar manner to the formation of sulphates of phenols and alcohols, the sulphate was derived from adenosine-3'-phosphate-5'-sulphato-phosphate (PAPS) via a specific arylamine sulphokinase. Roy (1960) also

indicated that this transferase was activated by the presence of certain 17-oxo steroids and suggested that the formation of a steroid enolsulphate, which may act as a sulphate donor, could occur under certain conditions in vivo.

The other general metabolic conjugation reaction of arylamines is the formation of acyl derivatives, ArNHCOR. As will be discussed in Section 4 the most common example of this type of conjugation is the formation of acetyl derivatives which involve the transfer of the acetyl group from acetyl Coenzyme A (acetylCoA) to the amino group. Although other acyl derivatives are commonplace in lipid biochemistry, few other acyl derivatives of aromatic amines have been reported as metabolites. Sulphanilamide is converted to a succinyl derivative in vitro by tissue extracts (cited by Williams, 1959) and 2-naphthylamine is excreted by rats and dogs as 2-formamido-1-naphthylsulphate (Boyland and Manson, 1966). The mechanism of these acylations has not been further investigated.

The reverse reaction, that is the deacylation of acetamido compounds, has been reported, and occurs with both aromatic and aliphatic compounds. At least two different enzymes, depending upon the nature of the substrate , are capable of carrying out this reaction. This hydrolytic reaction will be discussed in greater detail in Section 3. From the above it can be seen that when animals or man are exposed to aromatic amines both acetylation and subsequent deacetylation can occur, leading to an equilibrium

$$ArNH_2 \rightleftharpoons ArNHAc$$

As these two components have different physical properties, i.e. aromatic amines are basic whereas aromatic amides are not, it would not be surprising if they are substrates for different enzymes leading to different metabolic oxidation products.

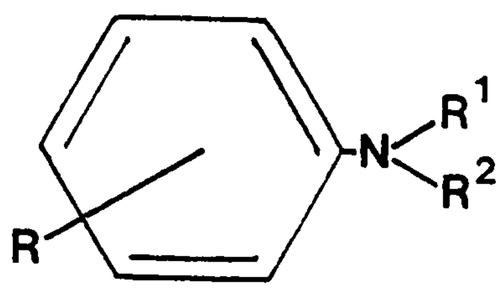


Fig.1.4.1. General structure of aromatic amines.

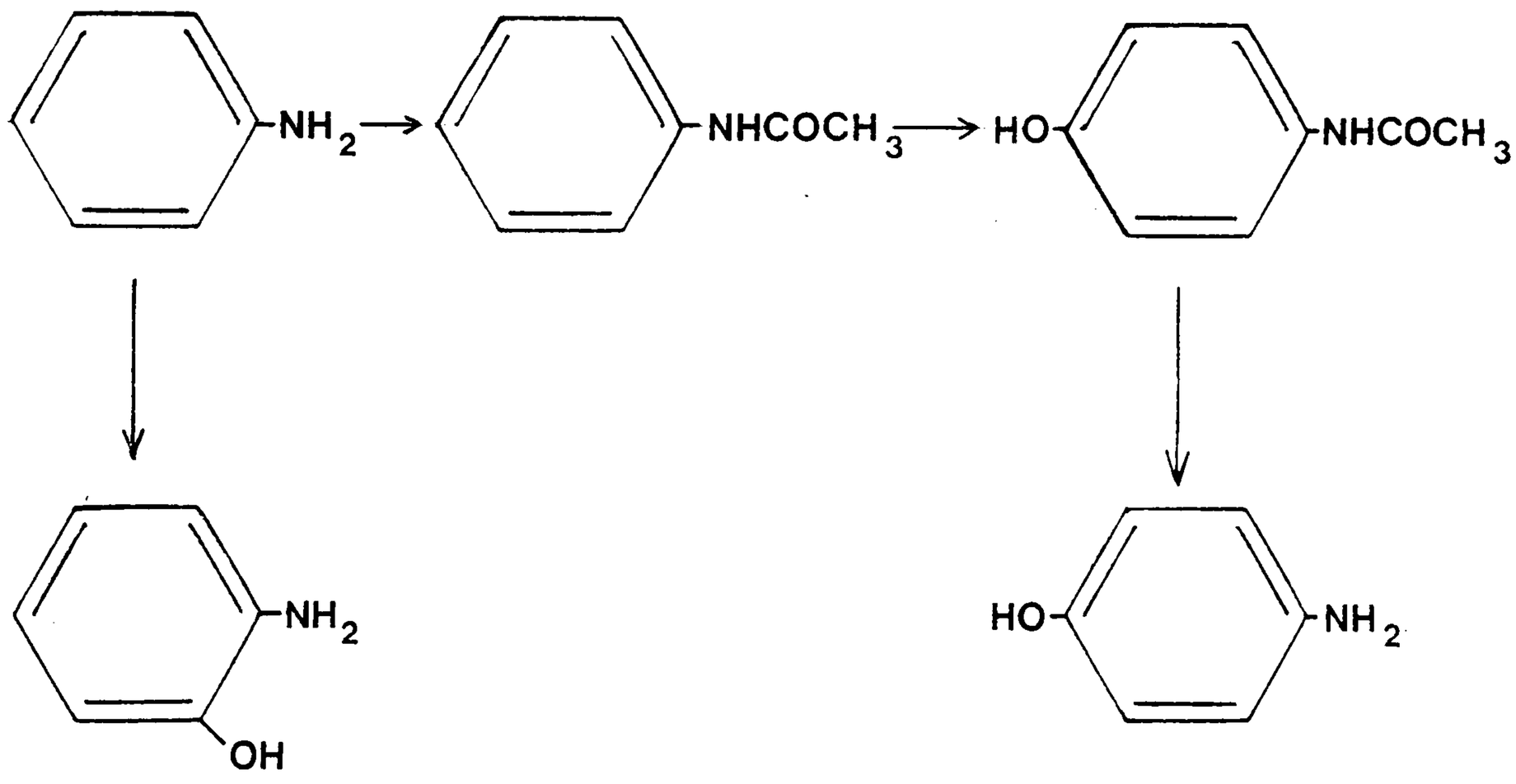


Fig.1.4.2. Possible involvement of acetylation in directing the site of hydroxylation of aromatic amines.

The nuclear parahydroxylation of aniline in vivo was recognised as a metabolic process (Schmiedeberg , 1878; Muller, 1887), and this site of hydroxylation was confirmed by Smith and Williams (1949a). These latter authors fed aniline to rabbits and found that the major metabolite was the acid-labile N-glucosiduronate; but that in addition about one-third of the dose was excreted as the sulphate derivatives of ortho- and para- aminophenol and 4-aminoresorcinol, indicating that ortho-hydroxylation was also possible. This work showed that the O-glucuronide fraction did not contain any conjugates of ortho-aminophenol although the glucuronide of para-aminophenol and para-acetamidophenol were formed. As earlier work on acetanilide metabolism in the rabbit had indicated that only parahydroxylation occurs (Smith and Williams, 1948), it seems that hydroxylation in the ortho-position can only occur with free aromatic amines. In contrast to the experiments above Jaffé and Hilbert (1888) fed acetanilide to dogs and found conjugates of both ortho-and para-aminophenol in the urine. These results suggest that the dog possesses a deacylase enzyme, although this species lacks an arylamine acetylase system. As aniline is converted in vivo to acetanilide in most species, the possibility arose that the para-aminophenol formed from aniline by rabbits, was derived by hydroxylation of acetanilide, and subsequent deacetylation, the ortho-aminophenol being formed by direct hydroxylation of the aniline (See Fig.1.4.2)

However, when aniline was given to dogs, a species which cannot acetylate aromatic amines, both ortho- and para-aminophenols were found as metabolites (Parke, 1960). In agreement with these findings, Clayson (1950) reported that when 2-naphthylamine was given to dogs, between 30 and 70% was excreted as conjugates of 2-amino-1-naphthol. Later studies using 8-^{14}C-2-naphthylamine indicated that the true figure for this route is probably in excess of 90% (Radomski, Brill and Deichmann, 1967).

In contrast, when 2-acetamidonaphthalene was given to dogs, less than 5% of the dose was hydroxylated in the 2-position. Similar results were obtained using the cat, indicating that both cat and dog had a high ortho-hydroxylase activity towards the parent amine, and a much lower activity towards the corresponding acetamido compound. With the latter compound hydroxylation to 2-acetamido-6-naphthol is the predominant route (Dobriner, Hofmann & Rhoads, 1941).

A considerable step forward in our understanding of the molecular mechanisms of aromatic amine metabolism came with the realisation that many of the metabolites which are formed in vivo, could be produced by suitably fortified tissue preparations in vitro. At first these preparations consisted of crude tissue minces or brei, but later tissue slices were used. Sato, Suzuki, Fukuyama and Yoshikawa (1955) studied the metabolism of aniline and acetanilide by liver slices prepared from rat, rabbit, dog and cat. In the same year Booth, Boyland and Manson showed that 2-naphthylamine was converted predominantly into 2-amino-1-naphthylsulphate, together with some 2-acetamido-6-naphthylsulphate and 2-amino-6-naphthylsulphate. However, as this preparation was able to acetylate and deacetylate aromatic amines and acetamido compounds it was difficult to establish the exact sequence of reactions leading to each metabolite.

Concurrent with this work, American scientists, notably those associated with Brodie, Axelrod and Udenfriend, established that a wide variety of drug oxidations could be carried out by microsomal fractions prepared from various tissues (See Brodie, Axelrod, Cooper, Gaudette, La Du, Mitoma and Udenfriend, 1955).

Using a microsomal system prepared from male New Zealand White rabbits Mitoma, Posner, Reitz and Udenfriend (1956) investigated the metabolism of both aniline and acetanilide. These authors found that aniline was converted only to para-aminophenol, whereas acetanilide was metabolised to all three isomers of acetamido-phenol, although the meta- and ortho-isomers were formed in only trace amounts. Surprisingly, these authors claimed that acetanilide was not hydrolysed to aniline by their preparation, whereas this process had been observed in the liver of many species, including rabbit, by Michel, Bernheim and Bernheim as early as 1937, and was later shown to be localised in the microsomal fraction.

These microsomal studies showed that the enzymes responsible for hydroxylating amines and amides required reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, and were inhibited by diethylaminoethyldiphenylpropylacetate hydrochloride (SKF 525A). A further study by Booth and Boyland (1957), using rat liver microsomes, showed that whereas acetanilide and 2-acetamidonaphthalene were hydroxylated at the carbon atoms furthest from the nitrogen substituent, the corresponding amines were hydroxylated also on the carbon adjacent to the nitrogen. In 1961 Posner, Mitoma and Udenfriend re-examined the site of hydroxylation of aniline by cat liver microsomes, and found that about equal amounts of both para- and ortho-aminophenol were formed, whereas this ratio was reported to be "large" when rabbit liver was used. These authors reported that the rates of para- and ortho-hydroxylation of acetanilide, were equal using either dog or cat

microsomes, whereas two hundred times more of the para-isomer was formed using rabbit liver microsomes. Thus the wide range of in vivo ratios of para- to ortho-amino phenols, previously reported in various species by Parke and Williams (1956), Parke (1960) and Piotrowski (1961), was to some extent verified in vitro.

Parke and Williams (1956) suggested that the two hydroxylated derivatives of aniline were formed by two separate hydroxylating enzymes. This idea has received further support from the work of Bauer and Kiese (1964) who showed that the enzymespresent in rabbit liver microsomes, which produced para- or ortho-aminophenol from aniline, responded differently to the effects of inhibitors incorporated into the incubation media. Thus, copper chloride or semicarbazide inhibited orthohydroxylation to a far greater extent than they did para-hydroxylation. In keeping with this greater sensitivity of the ortho-hydroxylase, it was found that storage of microsomes at 2-3°C for two weeks virtually destroyed this enzyme without affecting the parahydroxylase.

Kiese (1959a, 1959b) reported the presence of nitrosobenzene in the blood of dogs receiving aniline. This report is of fundamental importance in the study of the metabolism of aromatic amines as it clearly indicates that Kiese recognised that the aromatic amino-group was vulnerable to metabolic oxidation. This N-oxidative ability was shown to be localised in the microsomal fraction of liver preparations (Kiese and Uehleke, 1961) and required NADPH and molecular oxygen.

These observations led to a comparison of the processes involved in the metabolic N-oxidation and C-oxidation of aniline by liver microsomes, (Kampffmeyer and Kiese, 1963, 1964), with the conclusion that different

enzymes were involved in these hydroxylations. These conclusions were based primarily on the different effects of certain substances incorporated into the incubation system, e.g. whereas 2,4-dichlorophenol had no effect on N-oxidation of aniline, it inhibited p-aminophenol formation by about 70 %; a similar effect was observed after the incorporation of either D-penicillamine or α,α'-dipyridyl. Conversely semicarbazide or adenosine triphosphate enhanced the N-oxidation whilst inhibiting C-oxidation.

In these studies N-oxidation was only compared with the para-C-oxidation, whereas it was known that orthohydroxylation could occur. Bauer and Kiese (1964) concluded that not only are the ortho- and para-hydroxylation mechanisms different, but that these are both different from that involved in the N-oxidation of aniline.

This suggestion of three different hydroxylating mechanisms for these three sites of hydroxylation of aniline is at variance with the proposal of Miller and Miller (1960). These authors, after the observation that the carcinogen 2-acetamidofluorene was metabolised by attack on the amide nitrogen to yield the corresponding hydroxamic acid (Cramer, Miller and Miller, 1960) suggested that ortho-hydroxylation could arise via a rearrangement reaction. Such rearrangements are known to occur with arylhydroxylamines and hydroxamic acids chemically (Hughes & Ingold, 1952) and metabolically (Booth and Boyland, 1964; Gutmann and Erickson, 1969), but not under the conditions used by Bauer and Kiese (1964). This type of rearrangement has been invoked by Boyland, Manson and Nery (1963) to account for the metabolic formation of mercapturic acids from aromatic amines.

Further studies on the mechanism of these hydroxylations became possible following the discovery by Klingenberg (1958) and Garfinkel (1958), of a new cytochrome in microsomal preparations. This cytochrome, which was designated cytochrome P-450 by Omura and Sato (1962), was later shown to have a central role in the oxidation of drugs and foreign compounds by the endoplasmic reticulum. These studies showed that cytochrome P-450 acted as a terminal oxidase to an electron transport system which consisted of several components, the initial one being NADPH:cytochrome C reductase which was linked to cytochrome P-450 by a non-haem iron protein (Omura, Sato, Cooper, Rosenthal, and Estabrook, 1965). The simplicity of this system has been challenged, and the possible involvement of cytochrome b_5 in the transfer of a second electron has been proposed by Cohen and Estabrook (1971).

These proposed schemes for the electron transport systems involved in the microsomal oxidation of foreign compounds are shown in Figure 1.4.3.

From the above it can be seen that any procedure which interferes with any component of these chains could be expected to interfere with microsomal oxidations. Conversely any process which results in an increase in the rate-limiting component would be expected to enhance these oxidations.

Thus, generally, oxidations involving an attack on a carbon atom are inhibited by the presence of carbon monoxide in the atmosphere and enhanced by pretreatment of the animal with certain agents, e.g. phenobarbitone or 3-methylcholanthrene. Carbon monoxide forms a complex with cytochrome p-450 and prevents it participating in oxygen activation, whilst both phenobarbitone and 3-methylcholanthrene enhance the synthesis of microsomal haem from δ-aminolaevulinic acid.

Kampffmeyer & Kiese (1965) investigated the metabolism of aniline in the presence of a carbon monoxide plus oxygen atmosphere and found that, whereas the formation of p-aminophenol was inhibited, the oxidation on the aromatic nitrogen was unimpaired. This observation gave rise to a generalisation that nitrogen oxidation was different to that of carbon oxidation, the latter utilizing the cytochome P-450 system, whilst the nitrogen oxidising system seemed to be able to function without cytochrome P-450. This theory was strengthened when Ziegler, McKee & Poulsen (1973) showed that a purified amine oxidase from pig liver was able to N-oxidise aromatic amines. This purified flavoprotein amine oxidase was devoid of cytochrome P-450. However, the situation is more complex. Uehleke and Brill (1968) have reported an increase in the excretion of N-oxidation products of aromatic amines in animals which were pretreated with phenobarbitone, thus implicating the cytochrome P-450 system (as the flavoprotein is non-inducible).

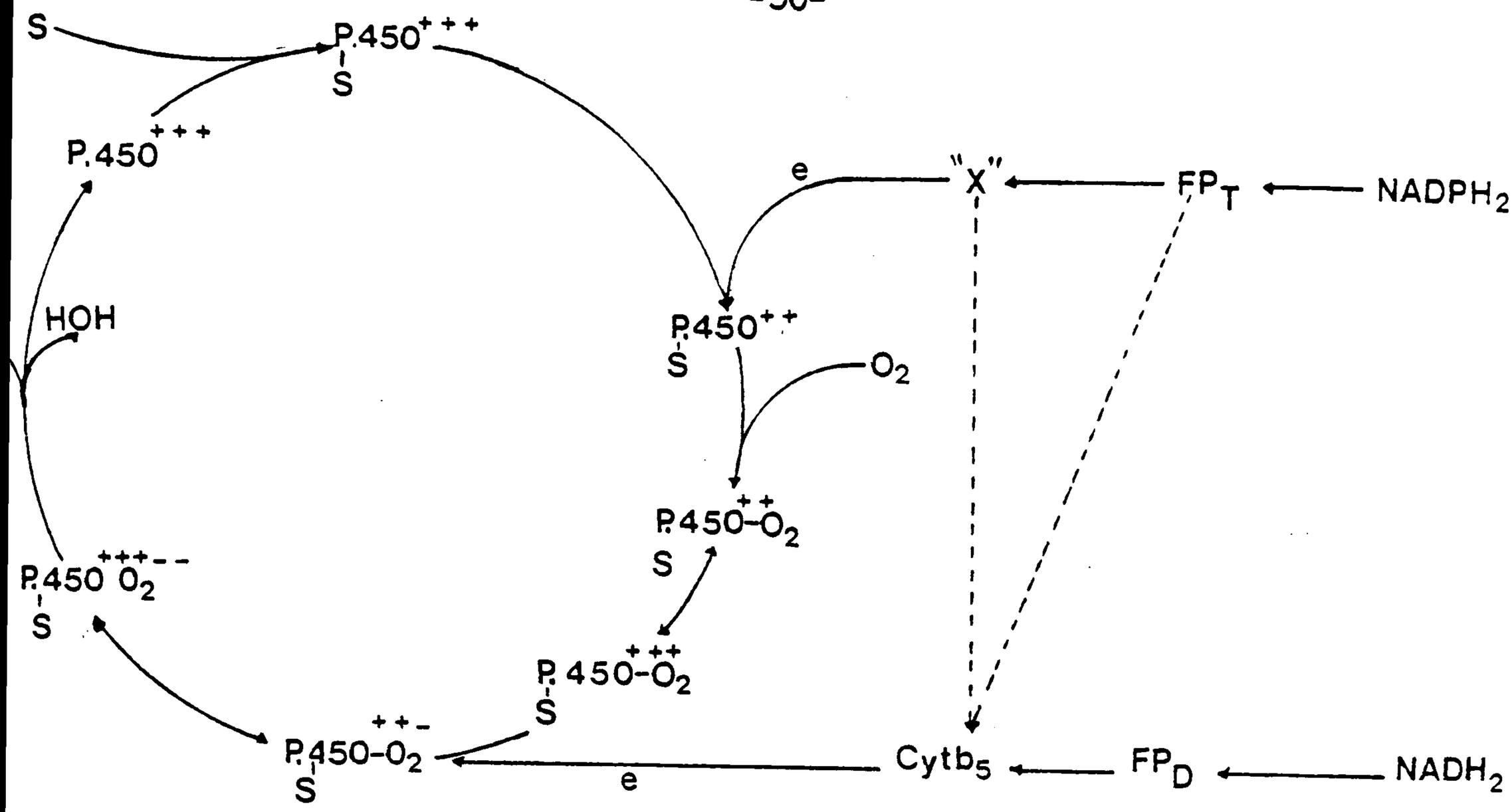
In an attempt to rationalise these and many other observations, Gorrod (1973) proposed that basic amines were predominantly N-oxidised by Ziegler's flavo-protein enzyme, whilst the non-basic amides were N-oxidised by the cytochrome P-450 system. As aromatic amines have pKa values intermediate between aliphatic amines and amides it was further proposed that they may very well be substrates for both enzymes yielding the same products by different mechanisms. The actual enzyme involved would then depend upon the species distribution of the enzymes and the affinity of the aromatic amine towards the individual oxidases.

Recently, Lotlikar, Wertman and Luha (1973) and Lotlikar, Luha and Zaleski (1974) have clearly shown that cytochrome P-450 is involved in the N-hydroxyla-tion of 2-acetamidofluorene. However, this molecular species of cytochrome P-450

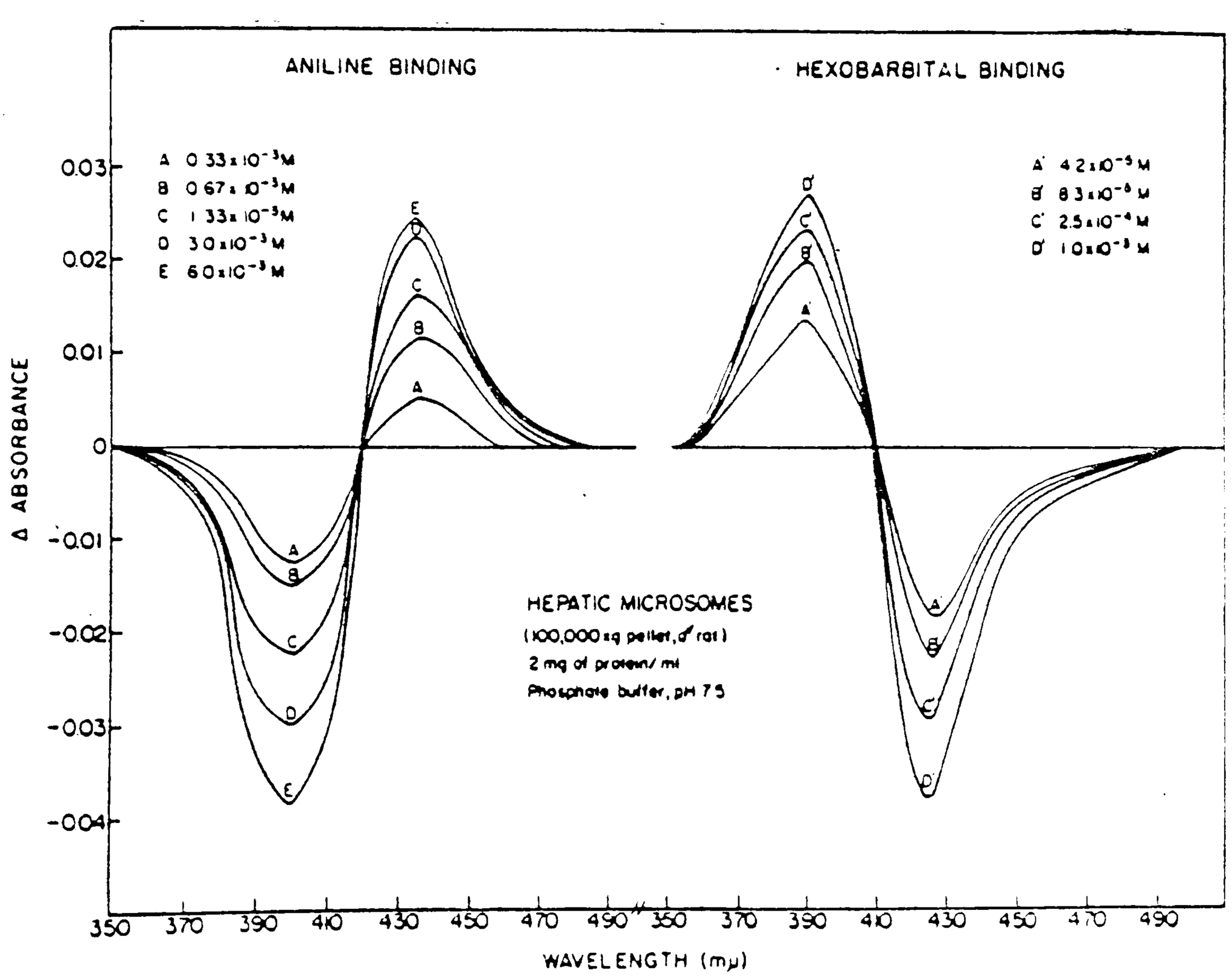
must be different to that involved in the oxidation of the carbon sites of 2-acetamidofluorene as this latter N-oxidative process is lacking in the guinea pig, a species well able to hydroxylate the other sites on the molecule.

In view of the foregoing discussion it seems peculiar that aniline p-hydroxylase is still used as a "marker" enzyme for studies on the mechanism of oxidation, or for investigating the influence of physiological or pathological parameters on drug metabolising enzymes. In these cases only the p-hydroxylase is measured, the other sites being completely ignored.

A further complication arises in our understanding of the microsomal mechanism of aromatic amine oxidation. It is known that substances capable of undergoing oxidation are capable of interacting with microsomal cytochromes to give either a Type I or Type II spectral change (Remmer, Schenkman, Estabrook, Sasame, Gillette, Narasimhulu, Cooper & Rosenthal, 1966) and that these changes are concentration dependent (Schenkman, Remmer & Estabrook, 1967). (Fig.1.4.4.). These latter authors have suggested that the Type I spectral change is caused by an interaction with the enzyme protein and/or lipid and results from a conformational change; the Type II change is thought to be the result of a direct interaction with microsomal haem at the same site as that normally occupied by oxygen. Primary aromatic amines all give a Type II spectral interaction (Temple, 1971) and this is thought to occur by donation of the lone pair of electrons to allow the base to act as the sixth ligand of the iron in the protoporphyrin. Support for the involvement of these electrons comes from the observation of Gorrod, Temple and Disley (1971) who showed that as the pKa of para-substituted anilines was reduced, so the spectral interaction with microsomal cytochrome at a fixed concentration (1mM) of



1.4.3. Scheme for microsomal electron transport system (after Estabrook, Franklin, Baron, Shigematsu & Hildebrandt 1971).



g.1.4.4. Type I (hexobarbital) and Type II (aniline) binding spectra (after Mannering, 1972).

amine, decreased.

If indeed, the nitrogen of aromatic amines is attached to the cytochrome iron, it is difficult to envisage how the oxygen is able to oxidise the molecule. A partial explanation of this problem was offered by Gorrod and Temple (1973), who, by the use of spectral modifiers,were able to show that the observed spectra of anilines were in fact composite spectra with at least two components. These authors suggested that the Type I component was an indication of the binding site which allowed oxidation. This observation would also explain the finding (Schenkman et al, 1971) that whereas substances which gave a Type I interaction gave a good correlation between the Km and Ks values, aniline (which gives a Type II interaction) did not.

It is also known that in vitro metabolism is greatly affected by the incorporation of certain other chemicals into the incubation media. Thus, acetone is known to enhance the parahydroxylation of aniline (Anders, 1968; Vainio & Hanninen, 1972), whereas alcohols are known to inhibit this reaction in vitro (Cohen & Mannering, 1973).

In addition, the aniline parahydroxylase activity is dependent upon the nutritional status of the animal (Wade, Wu & Lee, 1975) and can be greatly increased by pretreatment of the animals with urethane (Schenkman, Ritchie, Cha & Sartorelli, 1974).

At the present time, whilst a great deal of information has accrued regarding the metabolism of aromatic amines, there is still a paucity of knowledge regarding the molecular mechanism of their oxidation.

The metabolism of certain specific carcinogenic amines will be discussed in Section 1.6.

1.5 Spontaneous Bladder Cancer and Tryptophan

In previous sections the relationship between exposure to aromatic amines and cancer production in various biological systems has been established. In man the clinically recognised condition, after exposure to certain of these substances, was cancer of the urinary tract. Aromatic amines are used in various industries, and therefore, a certain proportion of the tumours recognised in a population can be related to industrial exposure. However, the majority of a population are not employed in these industries, nor exposed to the compounds discussed earlier, yet tumours of the urinary tract still develop. This fact suggested that if cancer of the urinary tract was produced by a metabolite of an exogenous amine then the "spontaneous" disease might be caused via an analogous endogenous compound.

In biological systems, few aromatic amines occur naturally; p-amino-benzoic acid is involved in the biosynthesis of folic acid and anthranilic acid is converted to tryptophan by some organisms. In man, urinary aromatic amines are derived mainly from the metabolic breakdown of tryptophan.

Early studies had shown that the site and frequency of tumour development was often dependent on the protein content of the animal diet, and that in some cases riboflavin supplements exerted a protective effect. It was against this background that Dunning, Curtis and Maun (1950a) reported on the influence of D L-tryptophan added to a synthetic diet containing twenty-five percent tryptophan-free casein hydrolysate, on the subsequent development of tumours in rats receiving diethylstilboestrol. These authors reported that supplementation of a diet with 1.4% D, L-tryptophan increased the number and percentage of induced

mammary tumours, and significantly reduced the average latent period. When the tryptophan supplement was increased to 4% the reverse effect was observed, viz, a decreased number and percentage of tumours and a significantly prolonged latent period. These same authors (1950b) extended their study to examine the influence of added tryptophan on the occurrence of liver and bladder cancer in rats, induced by 2-acetamidofluorene (0.06%) added to the diet. In this case, addition of 1.4% or 4.3% tryptophan to a tryptophan-free casein hydrolysate -containing diet, increased the number of liver tumours from 35% to about 70%. At the same time, all the rats receiving 1.4% tryptophan developed bladder tumours, as did 90% of those receiving 4.3% tryptophan supplement. The control rats which received equivalent casein, in addition to the 0.06% 2-acetamidofluorene, never developed bladder tumours.

In further experiments, Dunning & Curtis (1958) confirmed their earlier observations and showed that both indole and indoleacetic acid also had the ability to produce urinary bladder neoplasms in rats with diets containing 2-acetamidofluorene.

The effect of dietary tryptophan supplements on tumour production by nitrosamines has been studied by Japanese workers, and has given certain ambiguous results. Tryptophan appears to have a synergistic effect on the production of urinary bladder tumours in rats induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine (Okajima, Hiasa, Imoto, Hiramatsu and Ito, 1968), and on the production of hepatomas in rats receiving N-nitrosodiethylamine (Kawachi, Hirata and Sugimura, 1968). In another study by the former group (Okajima et al. 1971), no synergistic effect was found between supplementary

tryptophan and bladder tumour production in rats which were also fed the bladder carcinogen N-nitrosodibutylamine. In these experiments the tryptophan had a protective effect against the production of the liver tumours normally additionally found with N-nitrosodibutylamine.

The idea that tryptophan may protect the liver and encourage dietary hepatocarcinogens to be excreted in the urine, and thus produce their carcinogenic effects in the bladder, is considered by Weisburger, Hadidian, Fredrickson and Weisburger (1967).

More recently Radomski, Glass and Deichmann (1971) have shown that dogs fed a diet supplemented with six grams of D,L-tryptophan daily, developed marked focal hyperplasia of the transitional epithelium of the urinary bladder. This level of the amino acid increased the normal daily intake by about seven-fold, but did not produce carcinoma even though the diet was continued for seven years.

The metabolism of tryptophan is rather more complex than the metabolism of simple aliphatic amino acids, as in addition to deamination and decarboxylation, normally found with amino acids, ring hydroxylation and ring fission of the indole nucleus can occur.

These products can be further metabolised by a wide variety of reactions, as shown in the general scheme of Figure 1.5.1, to produce nicotinic acid and thence nicotinamide. This route, which is known as the tryptophan-nicotinamide pathway, produces several aromatic amines as intermediates. The first step in this pathway is the oxidative fission of tryptophan to formylkynurenine which is mediated via the enzyme tryptophan pyrrolase. This compound is rarely detected in biological systems since the formyl group is easily removed by

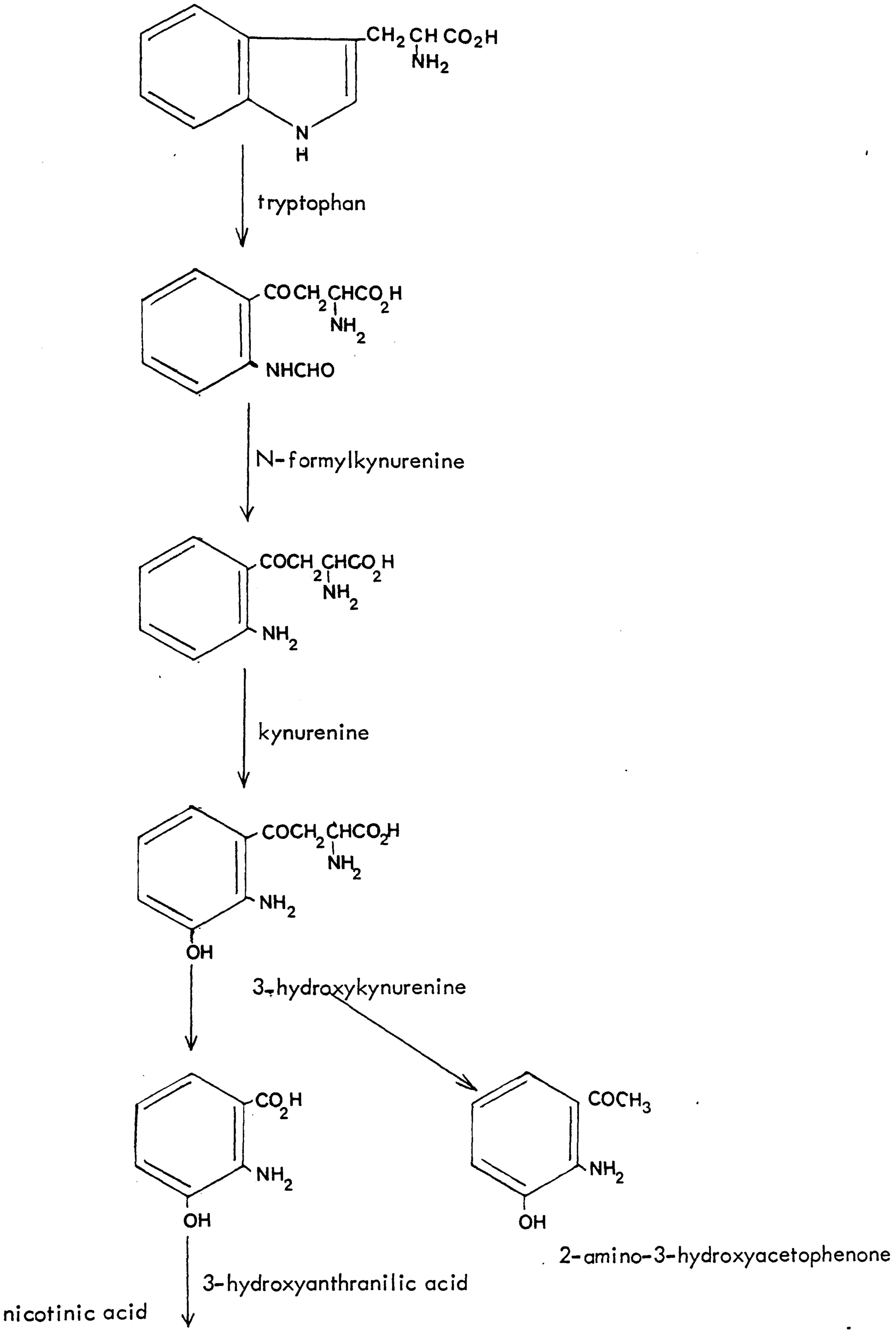


Fig.1.5.1. The metabolism of tryptophan to produce aromatic amines

kynurenine formamidase to produce kynurenine. Kynurenine is hydroxylated to form 3-hydroxykynurenine which is cleaved by kynureninase to yield 3-hydroxyanthranilic acid. This enzyme, which requires pyridoxal-5-phosphate, is also able to split kynurenine to anthranilic acid, although this appears to be a minor pathway. 3-Hydroxyanthranilic acid is then enzymically cleaved to form α-amino-β-carboxymuconic-ε-semialdehyde, which either spontaneously cyclises to give quinolinic acid, or is enzymically converted to picolinic acid and carbon dioxide (Nishizuka, Ichiyama, Gholson and Hayaishi, 1965).

In addition to this major pathway, tryptophan metabolites undergo many side reactions, thus kynurenine and 3-hydroxykynurenine are acetylated on the α-amino group, and both are substrates for a pyridoxal-5-phosphate dependent transaminase converting them to the corresponding α-keto-acids. These keto-acids then spontaneously cyclise to kynurenic acid and xanthurenic acid respectively. From the foregoing it can be seen that several aromatic amines are formed as metabolites of tryptophan. Additionally, Dalgliesh (1955) has indicated that 2-amino-3-hydroxyacetophenone is another aromatic amine, derived from tryptophan, which is sometimes present in human urine.

As early as 1941 some evidence indicated that this metobolic pathway could be involved in certain pathological conditions. Lepkovsky and Nielsen (1942) showed that pyridoxine-deficient rats excreted large amounts of xanthurenic acid. Otani, Nishino and Imai (1941) found that about 25% of patients with pulmonary tuberculosis excreted kynurenine when given 0.5g of tryptophan, whereas this substance was not found in control subjects under the same dosage regimen. However, it was not until the elucidation of the full metabolic

pathway and the development of a sensitive specific method for the analysis of these compounds that the role of tryptophan metabolism in certain conditions was established.

In 1952 Musajo, Spada and Coppini re-investigated the excretion of tryptophan metabolites in subjects with tuberculosis, and found that 11 out of 13 patients excreted 3-hydroxyanthranilic acid even without any tryptophan supplement. This observation was really the forerunner of many experiments designed to implicate 3-hydroxyanthranilic acid and some other tryptophan metabolites in disease states. Because of the variations in analytical techniques used (and other factors) some variation in the daily excretion of these compounds, in normal populations, was apparent (See Table 1.5.1).

Because of the wide variation, both in amounts and type of metabolite excreted, it is now common practice to supplement the subject's diet with tryptophan in this type of study. This is done with the intention of minimising dietary variations and saturating the enzymes utilising tryptophan metabolites as substrates. Results obtained under these conditions therefore more correctly represent the potential of an individual to utilise a particular metabolic pathway.

The earliest indication that endogenous aromatic amines could be involved in the etiology of non-industrial bladder cancer came from the observation of Ekman and Strömbeck (1947). These workers found unidentified diazotisable aromatic amines in the urines of bladder cancer patients. Later Boyland and Williams (1956) reported that urinary excretion of 3-hydroxyanthranilic acid was elevated in patients with bladder cancer. This observation was confirmed by Quagliariello, Auricchio,Casale & Tancredi (1958) and Tompsett (1959),

Table 1.5.1. The urinary excretion of some tryptophan metabolites in control populations

TRYPTOPHAN METABOLITE	Urinary excretion (mg/24 hr)						
	a	b	c	d	f*	g	h
kynurenine	2.5	3.1	1.14		14.5	15	-
N-acetylkynurenine	3.0	-	-	-	-	-	-
3-hydroxykynurenine	6.0	3.3	0.39	0.3-2.3	3.5	3	-
3-hydroxykynurenine-O-sulphate	-	-	-	-	-	7	-
kynurenic acid	2.1	-	2.83	-	-	-	-
xanthurenic acid	2.1	-	0.66	-	-	-	-
anthranilic acid	-	0.9	-	-	-	18	-
o-aminohippuric acid	4.7	-	0.62	-	-	-	-
3-hydroxyanthranilic acid	-	5.2	0.3	0.3-0.9[e]	-	30	1-15
3-hydroxyanthranilic acid-O-sulphate	-	-	-	-	-	12	-

a) Price (1958)

b) Tompsett (1959)

c) Bennassi, Veronese & Scoffone (1963)

d) Watanabe, Watanabe & Okada (1970)

e) Watanabe & Hayashi (1972)

f) Fletcher (1966)

g) Boyland & Williams (1956)

h) Rose & Toseland (1967)

* After subjects received 2g. L-Tryptophan

but other authors were not able to substantiate these findings. In Italy, Bennassi, Perissinotto and Allegri (1963) found that only 30% of bladder cancer patients excreted abnormal amounts of tryptophan metabolites. Kynurenine and 3-hydroxykynurenine were the most frequently elevated, whereas the 3-hydroxanthranilic acid level was abnormal in only 4 of the 201 cases studied. This finding is in contrast to that of Alifano, Papa, Tancredi, Elicio and Quagliariello (1964) who reported that 93% of their bladder cancer patients excreted elevated levels of 3-hydroxyanthranilic acid.

Other studies by Brown, Price, Satter and Wear (1960) showed that only about half of the patients they examined had abnormal tryptophan metabolism. These patients excreted more kynurenine, 3-hydroxykynurenine and acetylkynurenine than did a control population. 3-Hydroxyanthranilic acid was not measured in this study.

Treatment of these cancer patients with vitamin B_6 (pyridoxine) virtually normalised the urinary excretion of these metabolites. As pyridoxal phosphate is known to be a cofactor in both transaminase and kynureninase reactions, this result suggests that dietary deficiency may play a major role in the development of abnormalities of tryptophan metabolism. If this were so, it could account for the variations observed in subjects of different socio-economic backgrounds. However, it seems unlikely that vitamin B_6 deficiency would account for Boyland and Williams' (1956) observations, as their patients were selected from a well developed society on a North European diet.

The finding that certain of these tryptophan derivatives were carcinogenic or blastomagenic in certain systems, allowed the general concept that these

metabolites could be invoked in the induction of human bladder cancer. However, several observations are at variance with this idea. It is known that tryptophan metabolism is greatly influenced by the circulating levels of certain steroid hormones (Rose and McGinty, 1970) and hydrocortisone (Tenconi and Pasquariello, 1967). Therefore one might expect a correlation between physiological conditions which alter these levels and the production of bladder cancer.

Such a situation arises in a comparison between the sexes, and in the development of the individual. Rose (1967) has shown that young women excrete significantly greater amounts of 3-hydroxyanthranilic acid and 3-hydroxykynurenine than do males, yet females tend to have a much lower frequency of cancer of the bladder. In other work, Rose and Toseland (1967) and Toseland and Price (1969) have clearly shown that women taking oestrogen-progestogen preparations, as contraceptives, excrete between two and four times as much 3-hydroxyanthranilic acid as matched subjects not on these preparations. As yet there is no indication that women using oral steroid contraceptives are at a greater risk than those not on the 'Pill'. This aspect of tryptophan metabolism is further discussed in papers by Rose (1971) and Moursi, Abdel-Daim, Kelada, Abdel-Tawab and Girgis (1970).

Fletcher (1966) reported that the elevated excretion of 3-hydroxykynurenine was a secondary feature of many pathological conditions and no real difference could be associated with those patients suffering from a neoplastic disease. Similarly the inherited metabolic disorder, hydroxykynureninuria (Komrower, Wilson, Clamp and Westall, 1964) or the skin disease discoid lupus erythematos (Mandel and Appleton, 1964), in which high levels of 3-hydroxykynurenine are

excreted, are not considered premalignant states leading to bladder cancer.

However, despite these objections there still remains substantial evidence that aromatic amines derived from tryptophan play a role in human neoplastic disease. Studies in the Soviet Union by Raushenbach (1969) and Ivanova (1964) clearly implicate the involvement of 3-hydroxyanthranilic acid in adult leukaemia. In further studies on bladder cancer Yoshida, Brown and Bryan (1970) showed that bladder cancer patients with abnormal tryptophan metabolism develop recurrent neoplasms far more frequently than those patients with normal tryptophan metabolism.

It may be that further metabolism of certain of these compounds occurs and that these processes mask the importance of a certain metabolic route in a particular disease. It is known that 3-hydroxyanthranilic acid is converted to cinnabarinic acid in the urine of patients with bladder cancer (Nishimura, Pipkin, Duke and Schlegel, 1969), and is also conjugated with glucuronic and sulphuric acids (Watanabe and Minegishi, 1972; Watanabe, Ohkubo and Tamura, 1972; Watanabe, Minegishi and Tsutsui, 1972).

In view of the structural similarity between these endogenous compounds and known carcinogenic metabolites of carcinogenic aromatic amines (see Section 1.6), it seems likely that they are implicated in the spontaneous production of urinary bladder tumours. The elucidation of their total role in this disease awaits the development and application of new specific analytical methods.

1.6 Nature of "Active" Metabolites

Despite the early suggestions that hydroxylated metabolites of aromatic amines may be responsible for initiating the carcinogenic process following exposure to aromatic amines (Leuenberger, 1912 and Engel, 1920) it was not until 1953 that Clayson put forward the "orthoamino-phenol" hypothesis of amine carcinogenesis.

Clayson's hypothesis was based on his studies of 2-naphthylamine metabolism where he found that a good correlation existed between the percentage of administered 2-naphthylamine excreted as 2-amino-1-naphthol derivatives and the susceptibility of the species towards bladder cancer. This hypothesis gained further support when a number of aromatic amines and some of their hydroxylated derivatives were tested for local carcinogenic activity by direct implantation of the compounds into the lumen of the urinary bladders of mice (Bonser, Clayson, Jull and Pyrah, 1952; Boyland & Watson, 1956; Allen, Boyland, Dukes, Horning and Watson, 1957; Bonser, Boyland, Busby, Clayson, Grover and Jull, 1963; Boyland, Busby, Dukes, Grover and Manson, 1964). Essentially, these publications showed that under these conditions of testing most parent aromatic amines were virtually inactive, whereas the ortho-hydroxylated derivatives produced high yields of local tumours.

In addition, compounds which could produce "orthoamino-phenols" in situ by enzymic hydrolysis, e.g. 2-amino-1-naphthylglucosiduronic acid, or by oxidative dealkylation, e.g. 2-amino-1-methoxynaphthalene were also active. Compounds hydroxylated at positions other than ortho- to the amino group were inactive. It was during these studies that the "orthoamino-phenols" derived from tryptophan were shown to be carcinogenic.

The early results cited above led Boyland (1958) to review the biochemistry of amine carcinogenesis and suggest that the following sequence of events led to the release of the local active agent either in or adjacent to the bladder epithelial cells:

1. Amine absorbed and transported to liver
2. Amine hydroxylated and conjugated in liver
3. Aminophenol conjugate excreted in urine
4. Aminophenol conjugate enzymically hydrolysed.

As Boyland, Manson, Sims and Williams(1956) had found that sulphate conjugates of "orthoamino-phenols" were resistant to hydrolysis by various aryl-sulphatases, the glucosiduronic acid conjugates were considered to be the most likely precursor of the aminophenol.

In the mouse bladder implantation experiments, 2-amino-1-naphthol-glucosiduronate was even more active than 2-amino-1-naphthol hydrochloride in producing tumours. In 1958 Troll and Nelson detected a phosphate derivative of 2-naphthylamine in the urine of 2-naphthylamine treated dogs. This phosphate was subsequently synthesised and characterised as bis-2-amino-1-naphthylphosphate by Boyland, Kinder and Manson (1961) and later shown by bladder implantation to also be a carcinogen. Whilst both the glucosiduronic acid and phosphate derivatives of 2-amino-1-naphthol have been detected in those species susceptible to the carcinogenic effect of 2-naphthylamine, no phosphate conjugate has been detected which is derived from any other amine bladder carcinogen.

Further studies on the metabolism of 2-naphthylamine in various species did not produce the complete correlation between structure and activity which was hoped for. For example, the rabbit which does generate bladder tumours after exposure to 2-naphthylamine excretes very low levels of 2-amino-1-naphthol

conjugates; whereas the mouse which generates hepatomas and is resistant to bladder tumour formation excretes high levels of 2-amino-1-naphthol conjugates. Urine from this species contains adequate levels of β-glucuronidase to release 2-amino-1-naphthol (Boyland and Williams, 1960).

Another objection to the "ortho-aminophenol" hypothesis is that 1-naphthylamine is usually considered a non-carcinogen in most species, and yet 1-amino-2-naphthol conjugates are excreted. These would be expected to be hydrolysed to 1-amino-2-naphthol, a compound which is carcinogenic upon bladder implantation.

Conversely, a number of ortho-hydroxylated derivatives of known carcinogenic compounds were found to be inactive in mouse bladder implantation tests and when incorporated into the diet of experimental animals.

In 1960, Cramer, Miller and Miller discovered that the hepatocarcinogen, 2-acetamidofluorene was metabolically N-hydroxylated to form the corresponding hydroxamic acid. This was reminiscent of the N-oxidation of aromatic amines earlier described by Kiese (1959) and when N-hydroxy-2-acetamidofluorene was shown to be a powerful carcinogen, arylhydroxylamines became the logical candidates as the local carcinogen derived from aromatic amines. Indeed, 2-naphthylhydroxylamine produced tumours in the mouse bladder implantation test and subsequently Boyland, Dukes and Grover (1963) showed that it produced abdominal tumours after intraperitoneal administration to rats.

Since this time many N-hydroxyamides have been tested and shown to possess potent carcinogenic activity, but further work with arylhydroxylamines has again failed to convince that they are an absolute prerequisite as an intermediate in

amine carcinogenesis. Clayson (1965) reported that 2-naphthylhydroxylamine was inactive in skin painting experiments in mice and Boyland, Dukes and Grover (1964) showed that this compound failed to produce tumours when administered to guinea pigs in high doses. When it is remembered that many non-carcinogenic aromatic amines are also excreted as arylhydroxylamine metabolites, and that some of these have been shown to be highly carcinogenic or mutagenic in other systems, then their role in amine carcinogenesis is complex.

The further metabolism or reactivity of either ortho-aminophenols or aryl hydroxylamines may be important factors in deciding whether a tumour will ultimately be produced. In the case of ortho-aminophenols it has been proposed that a quinoneimine, produced by oxidation, is the reactive agent (Nagasawa and Gutmann, 1959). However, from 3-hydroxyanthranilic acid and 4-amino-3-hydroxybiphenyl, phenoxazones are easily formed by oxidation (Nagasawa, Gutmann and Morgan, 1959). Some phenoxazones have been tested and shown to possess carcinogenic activity. Aryl hydroxylamines can be oxidised in vivo to nitroso-compounds which in some cases are carcinogenic (Hecker, Traut and Hopp (1968) or they may rearrange to produce a reactive arylcarbonium ion (Boyland, Manson and Nery, 1963). Alternatively, nitrene (Rose, 1967) and nitroxide (Bartsch, Traut and Hecker, 1971) radicals have been suggested as being the local agent derived from aryl hydroxylamines.

The reactivity of some of these metabolites and their oxidation products with cellular macromolecules, and the possible role of these reactions in amine carcinogenesis will be discussed further in Section 9.

1.7 Aims of the Present Investigation

Although there have been many studies on the metabolism of aromatic amines and amides, few comparative studies of both an aromatic amine and its derived amide have been carried out using in vitro microsomal systems. One of the aims of the present investigation will be to examine the in vitro metabolism of the carcinogens 4-aminobiphenyl and 4-acetamidobiphenyl.

As some evidence in the literature suggests that amines and amides may be metabolised at different sites on the molecule, it is considered desirable to examine the biological acetylation of some amines and the deacetylation of the corresponding amides. In view of the "ortho-aminophenol hypothesis" of amine carcinogenesisand the resistance of the sulphate conjugates to enzymic hydrolysis, it is considered desirable to examine the species variation in glucuronyl transferase towards 4-amino-3-hydroxybiphenyl. This should give an indication of the potential amount of this glucuronide conjugate excreted in the urine of certain species, and may correlate with their susceptibility towards cancer of the urinary bladder, providing an active β-glucuronidase can be demonstrated in the urine.

As the dog is unable to acetylate aromatic amines, and as this species is susceptible to the carcinogenic action of some amines, it is thought that a hydroxylated derivative of the amine (rather than the amide) is likely to be the local carcinogenic agent. In order to gain evidence for or against this view it is proposed to carry out tests of the carcinogenicity of some hydroxylated derivatives of 4-aminobiphenyl.

A recent view on the mechanism of action of amine metabolites in initiating the carcinogenic process implicates the interaction of the metabolite with a specific cell organelle - the lysosome. As lysosomes vary in their properties, both from species to species and from organ to organ, any support for this idea would require the interaction of the active metabolite with lysosomes prepared from specific target organs. It is therefore proposed to investigate this hypothesis by examining the interaction of a series of carcinogenic and non-carcinogenic amines, and some hydroxylated derivatives, with a lysosomal preparation derived from the epithelial cells of the dog urinary bladder.

The results obtained from proposed investigations will then be discussed in relation to the overall situation of the mechanism of amine carcinogenesis.

SECTION 2 THE "IN VITRO" HEPATIC OXIDATIVE METABOLISM OF 4-AMINOBIPHENYL AND 4-ACETAMIDOBIPHENYL

2.1 INTRODUCTION

4-Aminobiphenyl and 4-acetamidobiphenyl are both compounds which have been shown to be carcinogenic in a variety of animal species. The former compound has also been implicated as a factor in the etiology of certain human bladder tumours produced in specific industrial environments (see sections 1 and 6). Various animal species differ in their susceptibility towards the carcinogenic effects of these compounds, and even in sensitive species, the carcinogenic response may be elicited in different organs, depending upon whether the animal was exposed to the amine or amide (for fuller discussion and details see Sections 1 and 6).

As these compounds produce their effects in organs anatomically distant from the site of administration, it is probable that a metabolite, rather than the parent compound, is involved in initiating the carcinogenic process. It was with a view to understanding the role of their metabolites in amine, or amide carcinogenesis that previous studies on the metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl were undertaken.

Bradshaw and Clayson (1955) identified 4-amino-3-biphenylyl sulphate, as the only metabolite detected, in the urine of dogs fed 4-aminobiphenyl, indicating that hydroxylation of the nucleus ortho to the amino group had occurred. This metabolite was also detected in the urine of most other species fed 4-aminobiphenyl (Bradshaw 1955, 1959). The percentage of the dose of 4-aminobiphenyl, excreted as 4-amino-3-biphenylyl sulphate in the urine, varied according to the species examined. The dog, rat, hamster and mouse excreted 40, 5, 2 and 1% of the dose respectively, as 4-amino-3-biphenylyl-sulphate, whereas in the urine of guinea pigs receiving 4-aminobiphenyl, no

4-amino-3-biphenylylsulphate was detected. The metabolism in vivo of 4-aminobiphenyl (Bradshaw, 1957, 1959) was further studied in the rabbit. This worker using paper chromatographic techniques, found no unconjugated 4-amino-3-hydroxybiphenyl or 4-amino-2'-hydroxybiphenyl or their N-acetyl derivatives were present in urine, whereas the rabbit excreted both 4-amino-4'-hydroxybiphenyl and 4-acetamido-4'-hydroxybiphenyl. Whether 4'-hydroxylation preceded N-acetylation or vice-versa, or whether direct 4'-hydroxylation of 4-aminobiphenyl occurs, could not be established in this in vivo study. Elson, Goulden and Warren (1958) had observed a considerable rise in the urinary excretion of glucuronic acid following administration of 4-aminobiphenyl to rats, so the possibility arose that 4-aminobiphenyl was excreted as conjugates of glucuronic acid as well as the sulphate ester detected by Bradshaw and Clayson (1955).

Studies on the metabolism of 4-acetamidobiphenyl in vivo (Wyatt, Miller & Miller, 1961; Miller, Wyatt, Miller & Hartmann, 1961) showed that both rats and dogs excrete N-hydroxy-4-acetamidobiphenyl as a glucuronic acid conjugate. The same metabolite was found in the urine after administering 4-aminobiphenyl to rats, but not when the amine was given to dogs. This latter finding is explicable in view of the inability of the dog to acetylate aromatic amines (see Section 4). In the urine of one dog fed 4-aminobiphenyl Miller, Wyatt, Miller and Hartmann (1961) detected a labile compound which they tentatively suggested was a conjugate of 4-hydroxylaminobiphenyl. These authors also report that the same metabolites were found in rat urine irrespective of whether 4-aminobiphenyl or 4-acetamidobiphenyl was fed to the animals, indicating to them the ease of acetylation of 4-aminobiphenyl by the rat.

The possible hydrolysis of 4-acetamidobiphenyl to 4-aminobiphenyl was not discussed. The metabolites detected were not fully characterised, but because of their reactivity with Folin-Ciocalteau reagent, and their ability to couple with diazonium reagents, they were thought to be phenolic compounds. By analogy with the known sites of hydroxylation of 2-acetamidofluorene it was suggested that hydroxylation had occurred at the 3, 2' and 4' position of 4-acetamidobiphenyl.

The finding of N-hydroxylated metabolites of carcinogenic aromatic amides (Cramer, Miller & Miller, 1960 and discussion above) stimulated investigations as to whether carcinogenic amines could also be N-oxidised. Kiese (1959a & b) had previously shown that aniline was converted to nitrosobenzene by cats and dogs in vivo. Troll and Nelson (1961) found N-hydroxy derivatives of 2-naphthylamine in the urine of dogs and patients exposed to the amine. 4-Aminostilbene (Miller & Miller, 1962) and benzidine (Troll, Belman & Rinde, 1963) were also found to be excreted in several species as N-hydroxylated metabolites.

Uehleke (1961a) was able to carry out the N-hydroxylation of aniline and some N-alkylanilines in vitro using rat hepatic microsomal preparations fortified with NADPH and oxygen. Uehleke (1961b, 1961c) extended his study to the N-hydroxylation of 2-aminofluorene and 2-naphthylamine in vitro. Irving (1962a & b) examined the N-hydroxylation of 2-acetamidofluorene in vitro by rabbit liver microsomes.

The N-hydroxylation of 4-aminobiphenyl in vitro was first observed by Uehleke (1963, 1964 a & b) using rat liver microsomes fortified with NADPH and oxygen, and in the same publications detected 4-nitrosobiphenyl in the blood of cats receiving 4-aminobiphenyl.

The first systematic study of the in vitro metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl was reported by Booth and Boyland (1964) using rabbit liver preparations. In this species hydroxylation of both 4-aminobiphenyl and 4-acetamidobiphenyl was observed using the microsomal system as enzyme source, however the site of nuclear oxidation varied depending upon the substrate. Metabolic hydroxylation of the nitrogen atom was observed using the rabbit microsomal system with both substrates. Whereas 4-aminobiphenyl was oxidised only on the carbon atom ortho to the nitrogen (3), in contrast 4-acetamidobiphenyl was only oxidised in the peri-para position (4').

Booth and Boyland (1964) also showed that both rat and rabbit hepatic soluble preparations, free of microsomal hydroxylating activity, were able to enzymically isomerise the N-hydroxylated derivatives of both 4-aminobiphenyl and 4-acetamidobiphenyl to -yield the corresponding ortho-hydroxylated amine and amide respectively. Further, these authors were able to show that rabbit microsomal preparations were able to hydrolyse 4-acetamidobiphenyl as well as N,-3- and 4'-hydroxy-4-acetamidobiphenyl. This microsomal deacetylating system was inhibited by the incorporation of potassium fluoride into the incubation medium. It became clear from this study that hydroxylation could appear to have taken place at a certain position on the 4-aminobiphenyl nucleus, whereas in practice it had either been hydroxylated in the corresponding position in 4-acetamidobiphenyl and subsequently hydrolysed, or hydroxylation of the nitrogen atom in the molecule and subsequent isomerisation and/or hydrolysis had occurred.

This work also showed that by the use of suitable precautions, such as using washed microsomal preparations, or by adding fluoride to the incubate, these secondary metabolic processes could be eliminated. The metabolic reactions of 4-aminobiphenyl and 4-acetamidobiphenyl observed by Booth and Boyland (1964) are shown in Figure 2.1.1.

The N-hydroxylation of 4-aminobiphenyl by hepatic rabbit microsomes in vitro was confirmed by Uehleke and Nestel (1967) who also showed that this reaction was carried out by rat liver microsomes, although at a slower rate than in the rabbit liver preparations. The N-hydroxylating activity of rabbit liver microsomes towards 4-aminobiphenyl was shown by these authors to be greatly enhanced if the animal had been pretreated with phenobarbital. Other sites of oxidation of the 4-aminobiphenyl molecule were not studied by Uehleke and Nestel (1967). The early indication that 4-hydroxylaminobiphenyl was present in dog urine after the administration of 4-aminobiphenyl (Miller, Wyatt, Miller and Hartmann, 1961) has been confirmed on several occasions (Fefer, Brill and Radomski, 1967; Radomski and Brill, 1970; Brill and Radomski, 1971; Radomski and Brill, 1971). More recently Radomski, Conzelman, Rey and Brill (1973) have shown that monkeys given 4-aminobiphenyl excrete 4-hydroxylaminobiphenyl as well as N-hydroxy-4-acetamidobiphenyl; in the dog the acetylated metabolite was not detected. In the ; dog 4-hydroxylaminobiphenyl was excreted as an acid labile glucuronic acid conjugate (Radomski, Rey & Brill, 1973).

A further minor metabolite of 4-acetamidobiphenyl has been recognised in which the acetamido group is oxidised to the corresponding glycolamide (Fries, Kiese & Lenk 1971, 1973). Further oxidation of this glycolamide to N-biphenylyloxamic acid has not been observed (Lenk, 1972)

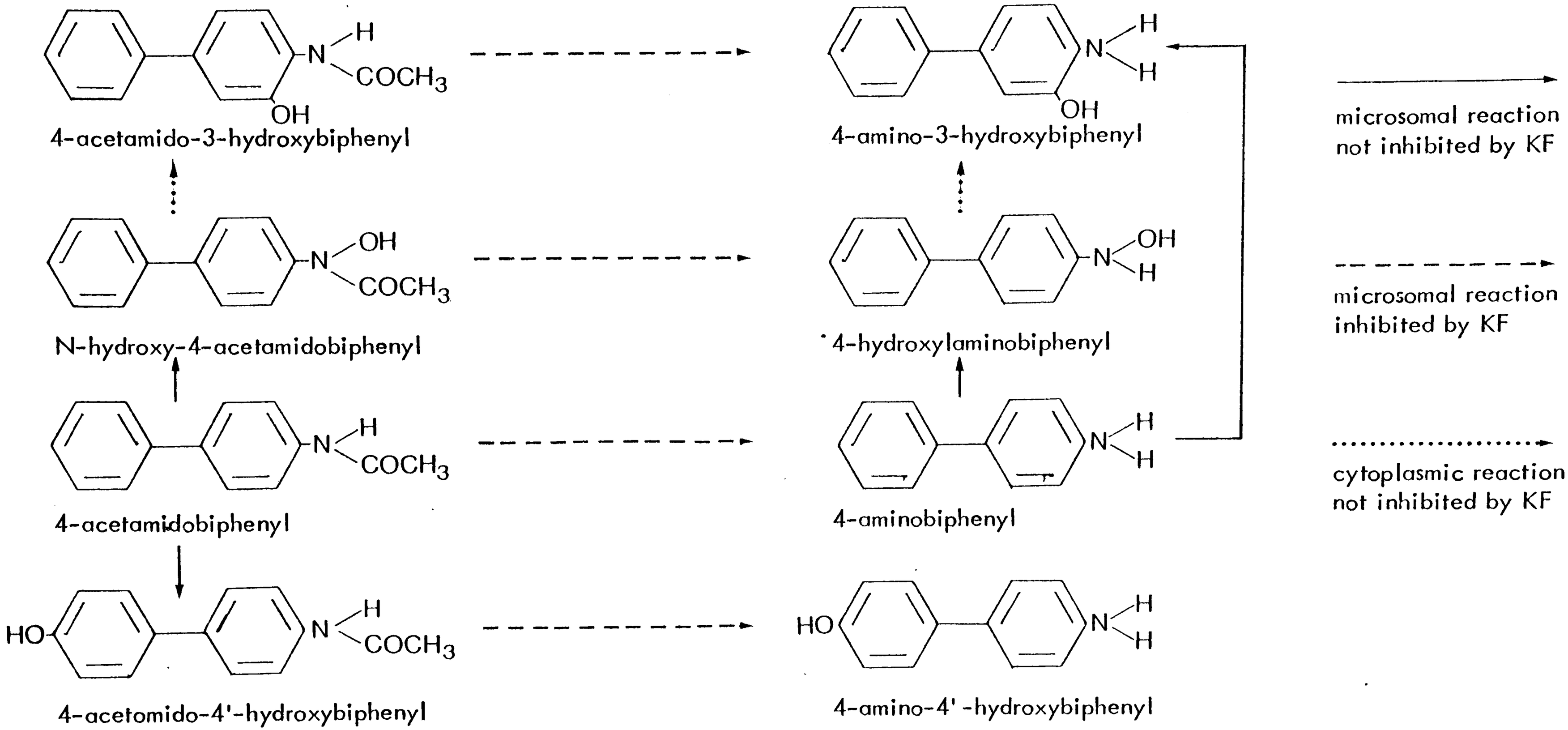


Fig.2.1.1. The in-vitro metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl by rabbit liver preparations (after Booth & Boyland, 1964).

In view of the known differences in species susceptibility to carcinogenesis by 4-aminobiphenyl and 4-acetamidobiphenyl, and the recognised differences of various species to hydroxylate compounds at different positions on a molecule, it is obviously desirable to extend the observations of Booth & Boyland (1964) to species other than the rabbit. In order to ascertain the relative importance of the various route of metabolism of 4-amino and 4-acetamidobiphenyl it is proposed to develop methods for the quantitation of their metabolites.

Previous methods used for the determination of arylhydroxylamines include reaction with salicylaldehyde plus potassium ferrocyanide or complexing with sodium pentacyano-ammine ferroate (Boyland & Nery, 1964) or diazotisation of the corresponding nitroso compound, formed by chemical oxidation, followed by coupling with a suitable amino (Herr & Kiese, 1959). Both and Boyland (1964) utilised the colour produced by the reaction between N-hydroxy-4-acetamido-biphenyl and ferric ions to measure the N-hydroxylation of 4-acetamidobiphenyl. In the proposed study, because of the desire to estimate the concentrations of individual metabolites in the presence of each other, it was decided to investigate the use of gas-liquid chromatographic methods as an analytical technique. Such techniques are known to have a high degree of specificity and sensitivity and are now widely used for drug metabolism and drug excretion studies.

Recently a new chromatographic technique has been developed in which the eluting solvent is passed at high pressurethrough the column. This technique, which is known as either High Pressure Liquid Chromatography or High Performance Liquid Chromatography (HPLC), has recently been applied to the separation of aniline and its metabolites (Sternson and De Witte 1977, 1978 and Sternson, De Witte and Stevens 1978) and a variety of nitrogen containing compounds and their N-oxidised derivatives (Nelson, Thorgeirsson and Wirth 1978). As this method is also sensitive, specific and non-destructive the use of this technique for the analysis of 4-aminobiphenyl and 4-acetamidobiphenyl metabolites will be investigated.

2.2 EXPERIMENTAL

2.2.1 Materials

2.2.1.1 Substrates, metabolites and related compounds *

4-Aminobiphenyl was obtained from Koch-Light Laboratories and shown to contain several impurities when examined by T L C or G L C (details of systems used are given later). Attempts at purification either by recrystallisation from benzene or aqueous ethanol in the presence of activated charcoal, or by sublimation at 100° under reduced pressure (15 mm), failed to completely remove the impurities. Pure 4-aminobiphenyl hydrochloride was obtained by hydrolysis of 4-acetamidobiphenyl with hydrochloric acid (2N) at 100°. The base was liberated from the salt by alkali, extracted into ether, the ethereal solution was washed, dried over sodium sulphate and evaporated to dryness at room temperature under reduced pressure. The 4-amino-biphenyl obtained gave a single discrete spot on T L C and a single peak with G L C systems. The mp of the purified amine was 54° in agreement with that found by Uehleke and Nestel (1967).

4-Acetamidobiphenyl was prepared by acetylation of impure commercial 4-aminobiphenyl with acetic anhydride in pyridine. The product was crystallised from ethanol and recrystallised from benzene-ethanol (95:5 by volume). 4-Acetamidobiphenyl was obtained as white needles, m.p. 171°. Hübner and Osten (1881) record a m.p. of 167° for this compound.

4-Amino-3-hydroxybiphenyl was prepared by the reduction of 4-nitro-3-hydroxybiphenyl with hydrazine hydrate in absolute alcohol in the presence

* yields were approximately the same as recorded in literature unless otherwise stated.

of palladium on charcoal at 50° for fifteen hours, using the general technique described by Kuhn (1951). 4-Amino-3-hydroxybiphenyl was recrystallised from aqeuous ethanol; m.p. 182° in agreement with Bradshaw (1958) and Boyland and Sims (1954).

4-Nitro-3-hydroxybiphenyl was obtained by treating 4-nitrobiphenyl (Koch-Light Laboratories) with finely-divided potassium hydroxide in benzene under reflux for three hours as described by Dermer and Drucker (1942). 4-Nitro-3-hydroxybiphenyl was recrystallised from ethanol; m.p. 103° in agreement with Colbert, Meigs and Jenkins (1937).

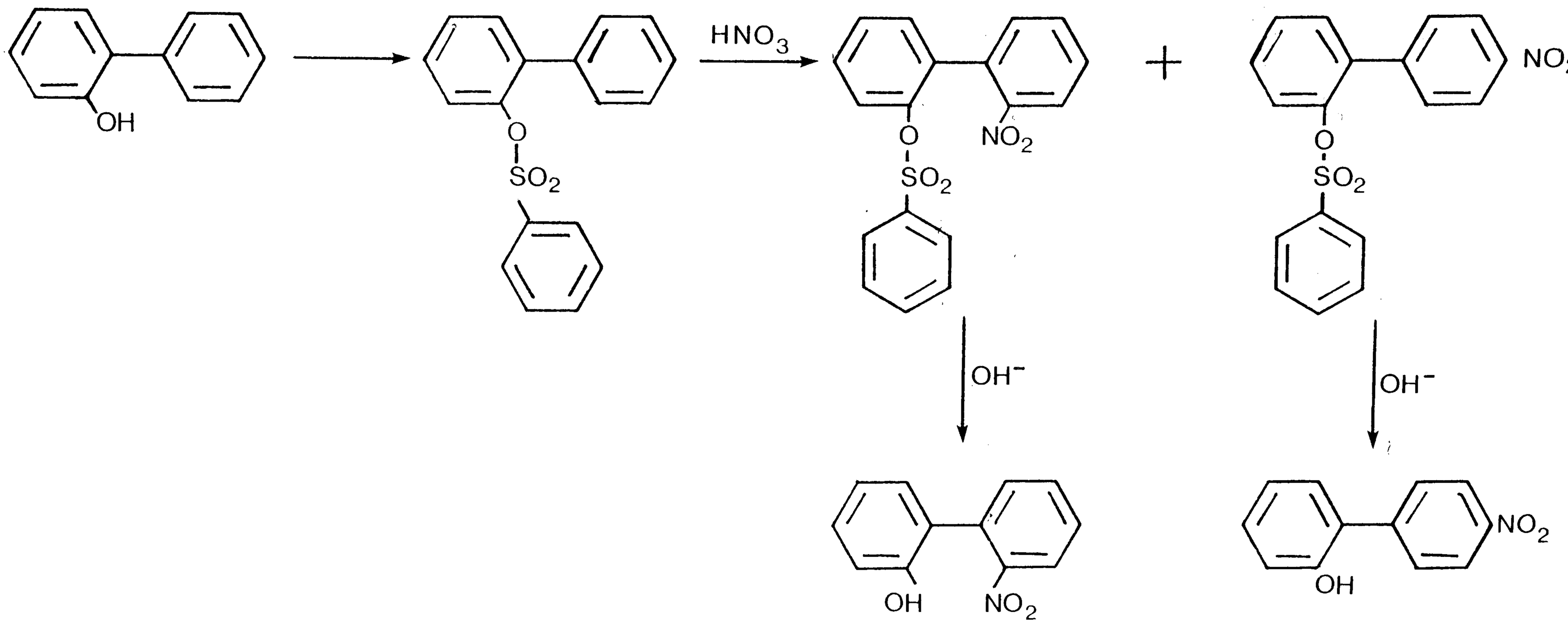
4-Amino-4'-hydroxybiphyl was prepared by reduction of 4-nitro-4'-hydroxybiphenyl using the hydrazine hydrate technique. 4-Amino-4'-hydroxybiphenyl was recrystallised from aqueous ethanol to give needles, m.p. 271°. Bradshaw (1958) records a m.p. of 269° for thiscompound

4-Nitro-4'-hydroxybiphenyl was prepared by the nitration of 4-biphenylyl benzoate followed by alkaline hydrolysis, as described by Jones and Chapman (1952). 4-Nitro-4'-hydroxybiphenyl melted at 200° in agreement with the literature.

4-Amino-2'-hydroxybiphenyl was prepared by reduction of 4-nitro-2'-hydroxybiphenyl in absolute alcohol using the hydrazine hydrate technique. 4-amino-2'-hydroxybiphenyl was recrystallised from absolute alcohol to give white needles, m.p. 191°. Despite being mentioned by Bradshaw (1958) its m.p. was not found in the literature. The analytical data is given in Table 2.2.1.

4-Nitro-2'-hydroxybiphenyl The synthesis of this compound was attempted using the route described by Colbert and Denny (1959) and outlined in

a)



b)

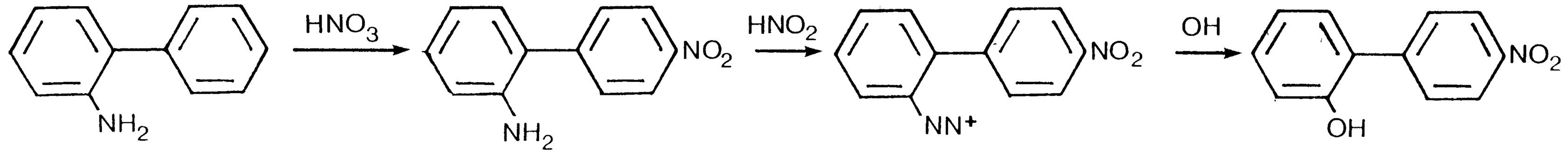


Fig. 2. 2. 1 Routes to the preparation of 4 - nitro - 2' - hydroxybiphenyl.

Fig. 2.2.1(a). However, despite several attempts the yields remained poor, multiple products were formed and separation of the required compound was extremely difficult.

4-Nitro-2'-hydroxybiphenyl was obtained via 4-nitro-2'-aminobiphenyl followed by diazotisation and reaction with hot dilute sulphuric acid as described by Harris and Christiansen (1933), Fig. 2. 2.1(b). Confirmation of the position of substitution of the nitro group on the nucleus was obtained by oxidation of the isomeric nitro-2'-hydroxybiphenyls and comparison of the products with authentic nitrobenzoic acids using thin layer chromatography.

4-Nitro-2'-hydroxybiphenyl was recrystallised from aqueous alcohol to form nearly white needles, m.p. 124° in agreement with Harris and Christiansen (1933).

4-Hydroxylaminobiphenyl was prepared by reduction of 4-nitrobiphenyl by aluminium amalgam as described by Bell, Kenyon and Robinson (1926). The product was recrystallised from benzene as pale yellow glistening leaflets, m.p. 152° in agreement with the literature.

4-Nitrosobiphenyl was prepared by the oxidation of 4-hydroxylamino-biphenyl with potassium permanganate in ethanol. The reaction mixture was filtered and chromatographed using an alumina column. The bright green material was eluted using petroleum ether (bp 40°-60°), which was removed by evaporation at 25° under reduced pressure. The product was crystallised from absolute alcohol to give crystals of 4-nitrosobiphenyl, m.p. 73°. Mijs (1959) and Uehleke and Nestel (1967) have previously described this compound. Attempts at the preparation of this compound by the direct oxidation of 4-aminobiphenyl with hydrogen peroxide, as suggested by Holmes and Bayer (1960), gave only 4-azoxybiphenyl,

mp 208°. Bell, Kenyon and Robinson (1926) record a mp of 212° for 4-azoxybiphenyl.

4-Acetamido-3-hydroxybiphenyl, 4-acetamido-2'-hydroxybiphenyl, 4-acetamido-4'-hydroxybiphenyl, 4-acetamido-N-hydroxybiphenyl were obtained by treating the parent aminophenol with acetic anhydride in pyridine at 50° for 20 minutes, followed by pouring the reaction mixture onto crushed ice. The N, O-diacetyl derivatives which were obtained were extracted into diethyl ether which was subsequently washed with hydrochloric acid (2N) and water. The ethereal extract was dried with sodium sulphate. Evaporation of the ether at 25° under reduced pressure yielded the N, O-diacetyl compounds which were recrystallised from aqueous ethanol as white crystals with the following melting points:-

4-acetamido-3-acetoxybiphenyl	135°
4-acetamido-2'-acetoxybiphenyl	145°
4-acetamido-4'-acetoxybiphenyl	226°
4-acetamido-N-acetoxybiphenyl	120°.

Bell Kenyon and Robinson (1926) record 119° for the latter compound. The required compounds were prepared from the N, O-diacetyl compounds by treatment with 1N sodium hydroxide in 50% aqueous ethanol at 60° until solution was effected. The solutions were filtered into the calculated amount of hydrochloric acid to neutralise the alkali, whereupon the corresponding 4-acetamido- N- or ring-hydroxy compounds were precipitated. The acetamido compounds were recrystallised from aqueous

alcohol to give white needles having the following melting points: 4-acetamido-3-hydroxybiphenyl 191°, in agreement with Miller, Sandin, Miller and Rusch (1956); 4-acetamido-4'-hydroxybiphenyl 224°, in agreement with Raiford and Clark (1926); 4-acetamido-N-hydroxybiphenyl 144°, in agreement with Miller, Wyatt, Miller and Hartmann (1961).

4-Acetamido-2'-hydroxybiphenyl, mp 145°, and all the ring acetoxy compounds appear to be new compounds. The analytical data obtained from elemental analysis is shown in Table 2.2.1.

N-(4-biphenylyl)-glycolamide and N-(4-biphenylyl)-oxamic acid were gifts from Dr. W. Lenk, Department of Pharmacology, University of MÜnich, Germany.

2.2.1.2. Cofactors

Glucose 6-phosphate (G6P), isocitric acid (ICA), glucose 6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (ICDH), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD) and their reduced forms (NADPH and NADH) were obtained from Boehringer Biochemicals Limited.

2.2.1.3. Inhibitors, Inducers and miscellaneous chemicals

Certain substances used as potential inhibitors, activators or inducers of microsomal oxidation were obtained from the following sources:

2,4 dichloro-6-phenyl-phenoxyethylamine (DPEA) was a gift from Dr. R. E. McMahon, Lilly Research Laboratories, Indianapolis, Indiana;

Table 2.2.1 Results of elemental analysis of new compounds described in Section 2

COMPOUND	mp	FOUND			REQUIRED		
		C%	H%	N%	C%	H%	N%
4-amino-2'-hydroxybiphenyl	191°	77.23	6.15	7.40	77.84	5.94	7.57
4-acetamido-2'-hydroxybiphenyl	209°	73.85	5.78	6.20	74.00	5.72	6.17
4-acetamido-2'-acetoxybiphenyl	145°	71.47	5.70	5.26	71.37	5.58	5.20
4-acetamido-4'-acetoxybiphenyl	226°	71.19	5.62	5.19	71.37	5.58	5.20
4-acetamido-3-acetoxybiphenyl	135°	71.09	5.65	5.26	71.37	5.58	5.20

7,8-benzoflavone (BF) from Dr. H. Gelboin, National Cancer Institute, Bethesda, Maryland; 1-methyl-2-mercapto-imidazole (methimazole, MMI) from Dr. D. M. Zeigler, University of Austin, Texas; 1,4-diazabicyclo (2,2,2)octane (DABCO) from Dr. U. Breyer, Institute of Toxicology, Tübingen, Germany. Arachlor 1254 was a gift from Monsanto Chemical Company, St. Lous, Missouri; andbromazepamfrom Dr. R. Long, Roche Products, Welwyn Garden City. 3-Methylphenanthrene was a gift from Dr.PSims, The Chester Beatty Research Institute, London; and 2-amino-3-hydroxyfluorene was purchased from Aldrich Chemical Company. Metyrapone was a gift from Ciba-Geigy Ltd., Horsham. 2-Diethylamino-ethyl-2,2-diphenyl valerate (SKF 525A) was a gift from Smith Kline and French Laboratories Ltd.; methylcholanthrene was obtained from Sigma Chemical Company; carbon monoxide and phenobarbitone from British Drug Houses Ltd. Other chemicals used for the preparation of buffers or isolation media or during analytical procedures were obtained from either British Drug Houses Ltd. or Hopkin and Williams Ltd. or Fisons Laboratory Chemicals Ltd.

2.2.1.4 Animals

All animals used were mature males of the following species and strains: rats, Wistar (200-300 g); guinea pigs, Duncan Hartley (300-500g); rabbits, New Zealand White (2.5-3 kg); mice, C.B.R.I. stock or C57 (25-35g); hamsters, Syrian (80-100g); dogs, Alsatian or Greyhound.

2.2.2 Methods

2.2.2.1 Tissue Preparations

Animals used, with the exception of dogs, were stunned and decapitated and allowed to bleed until heartbeat was no longer detectable. The liver was removed and, where applicable, the gall bladder excised. Any fat or large amounts of connective tissue were removed and the hepatic lobes were rinsed with ice-cold isotonic potassium chloride buffered to pH 7.4 with tris (tris/Kcl). The liver was blotted dry on filter paper, weighed and cut into small pieces under ice-cold buffered tris/KCl (two ml per gram of liver). Homogenisation was effected using a glass homogeniser fitted with a teflon pestle (Arthur Thomas, Size B) driven by an electric motor at 2,800 rpm. In the case of dog and rabbit liver preliminary homogenisation was carried out using an overhead drive homogeniser for 3 x 20 seconds with 5 second intervals at 0°. The teflon-glass homogeniser was kept in ice whilst it was being used; the tissue suspension was made to pass the pestle three times during the preparation. The homogenate was centrifuged at 10,000 xg for twenty minutes using a 8 x 50 ml rotor in a refrigerated Sorvall R C 2B centrifuge in order to sediment any residual whole cells, cell debris, nuclei, mitochondria and lysosomes. The supernatant was carefully collected and the microsomal fraction prepared by further centrifugation at 140,000 x g for 60 minutes using an 8 x 25 ml rotor in a M.S.E. Superspeed Model 40 or Model 50 centrifuge. The microsomal fraction was collected and resuspended in tris/KCl at a

concentration equivalent to 0.5 g of original tissue per ml of suspension. Dog liver was obtained from the Royal Postgraduate Medical School, Hammersmith, from dogs under general anaesthesia being used as donor animals in lung transplants. In these experiments the dog livers were placed in tris/KCl and surrounded by crushed ice for transportation to the Hammersmith annexe of Chelsea College.

2.2.3.2 Incubation Procedures

Incubations were normally carried out in open 25 ml Ehrlenmeyer flasks at 37± 1° in a shaking incubator (Gallenkamp). Incubates contained tissue equivalent to 0.5 g of liver in 1.0 ml of tris/KCl, substrates (5 μmol), glucose 6-phosphate (10 μmol), glucose 6-phosphate dehydrogenase (3 units), magnesium chloride (60 μmol) in a total volume of 3.5 ml. The substrates were added in 0.2 ml of methoxy-ethanol, and incubates in which 4-acetamidobiphenyl was used as a substrate also contained sodium fluoride (100 μmol) to inhibit deacetylation (see Section 3).

In experiments performed in conjunction with HPLC, incubations were carried out in 50 ml Ehrlenmeyer open-necked conical flasks in a shaking incubator at 37°. Each incubate consisted of tissue equivalent to 250 mg of original isocitrate (10 μmol) NADP (2.5 μmol) and isocitrate dehydrogenase (25 μl). In these experiments the substrate (2.5 μmol) was added in dimethylsulphoxide (25 μl).

2.2.2.3. Pretreatment of Animals with Inducing Agents

Animals were pretreated with potential inducing agents according to

the following dosage schedule. Phenobarbitone was given for three days at 80 mg/kg, methylcholanthrene for one day at 20 mg/kg and Arachlor 1254 for one day at 100 mg/kg. All substances were given intraperitoneally. The animals were sacrificed on the fourth day after the first dose of phenobarbitone, the fifth day after the dose of Arachlor and the third day after the dose of methylcholanthrene.

2.2.2.4 Experiments Involving Potential Inhibitors or Activating Agents

Potential inhibitors or activating agents were incorporated into the incubation medium at a final concentration of 10^{-3}M. In some cases solution or a suspension was obtained only after treatment in an ultrasonic bath prior to incorporation into the incubation medium.

2.2.2.5 Protein Determination

Microsomal protein was determined by the method of Lowry, Roseborough, Farr and Randall (1951) as modified by Miller (1959), using bovine serum albumin (BDH) as protein standards.

2.2.2.6 Cytochrome b_5 Determination

Microsomal suspension (1 ml) diluted with tris/KCl buffer (4 ml) was divided equally into two 1 cm glass cuvettes. Using a Unicam S P 800 spectrophotometer fitted with a scale expansion device a base line was established between 390 and 600 nm. The cytochrome b_5 in the sample cuvette was reduced with NADH (5 μl of a 2 μM solution) and the difference spectrum obtained between 390 and 600 nm.

2.2.2.7 Cytochrome P-450 determination

The cytochromes in both cuvettes, from the previous determination, were separately reduced by the addition of sodium dithionite (5 mg). One cuvette was then treated with a stream of carbon monoxide, delivered through a capillary tube to the bottom of the cuvette, for about 30 seconds. The difference spectrum of the reduced carbon monoxide-cytochrome complex against the reduced cytochrome was recorded between 390 and 600 nm.

2.2.2.8 Chromatographic Methods

2.2.2.8.1 Paper Chromatography

Bradshaw (1959) reported that he had used the reversed phase technique of Gasparic, Petránek and Večeřa (1955) for the successful separation of some hydroxy derivatives of 4-aminobiphenyl and 4-acetamidobiphenyl. Bradshaw pointed out that the method was associated with numerous difficulties which indicated that it may not be useful for routine use.

Whatman No. 1 paper was impregnated with formamide by passing the paper through a solution of formamide (10%) in absolute ethanol and allowing the paper to air dry at room temperature. Chromatograms were run by the ascending technique in closed glass tanks at room temperature for a maximum of 15 cm. The solvents used were: ethylene dichloride; toluene:ethylene dichloride 3:5 by vol. or toluene:ethylene dichloride 1:1 by vol.

2.2.8.2 Thin Layer Chromatography (TLC)

Thin layer chromatography was usually carried out using either Silica gel G or Silica gel G/UV254 (Machery Nagel) supported on glass plates

at a thickness of 0.25 mm. After speading, the plates were allowed to air dry, activated by heating to 100° for 45 minutes and cooled before use. In certain experiments aluminium oxide or cellulose was used spread on glass plates at a thickness of 0.25 mm. In these cases the alumina plates were activated at 100° for 60 minutes and the cellulose plates were heated at 100° for 10 minutes and cooled before use. All thin layer plates were stored in an air-tight cabinet over a desiccant prior to use.

2.2.2.8.2.1 Solvent systems for TLC

During the course of this work numerous solvent systems were tried in an attempt to find one which would separate all the hydroxylated derivatives of both 4-aminobiphenyl and 4-acetamidobiphenyl from the parent compounds. Usually the solvents were prepared by mixing various proportions of a non-polar constituent such as benzene, petroleum ether or cyclohexane with a polar compound such as acetone or an aliphatic alcohol. The compositions of certain solvent systems used for TLC are shown in Table 2.3.1.

The chromatographic properties of 4-aminobiphenyl and 4-acetamidobiphenyl together with compounds prepared as potential metabolites, using a number of solvent systems, are recorded in Table 2.3.2.

2.2.2.8.2.2 Detection reagents used in conjunction with either paper or thin layer chromatography

With the exception of ultra-violet light the detection reagents used were chosen because of their ability to react with a specific function of a molecule. For example, diazonium compounds were used to detect phenols; conversely,

aromatic amines were converted to diazonium compounds in situ on the chromatograms and detected by reacting with phenols. Arylhydroxylamines were detected by reagents which utilised the strong reducing properties of this group of compounds or their ability to form co-ordination complexes. The abbreviations used when any detecting reagent is referred to later in the text are shown in brackets, e.g. ultra-violet light (UV 254). Ultra-violet light: Chromatograms were viewed under ultra-violet light produced by a Hanovia portable fluorescence lamp (model CHI/294 or model 16). The lamps were fitted with suitable filters such that light of principally 254 nm (UV 25) or 366 nm (UV 366) respectively was emitted. Diazotisation and coupling in situ: chromatograms were lightly sprayed with hydrochloric acid (0.5 N), air dried for ten minutes and sprayed with sodium nitrite (0.5%). The diazonium compounds produced were coupled by spraying with an alkaline solution of one of the following phenols (0.5%) in sodium hydroxide (1N) in aqueous ethanol (50%). The phenols used were 1-naphthol (1-N), 2-naphthol (2-N), 4-hydroxybiphenyl (4-HB), 2-hydroxybiphenyl (2-HB) and hexylresorcinol (HR). p-Dimethylaminobenzaldehyde (DAB) and p-dimethylaminocinnamaldehyde (DAC) were used as a 0.5% solution in ethanol containing concentrated hydrochloric acid (1 ml/100 ml). Diazotised sulphanilic acid (DSA) and diazotised p-nitroaniline (DNA) were prepared immediately before use by treatment of the parent amine (0.2% in 0.5 N HCl The commercially-available stable diazonium compound, 2-chloro-4-nitrobenzene diazonium naphthalene-2-sulphonate (NNCD) (Hopkin and

Wi lliams Limited) was used as a 0.2% aqueous solution. After treatment with the diazonium reagents the chromatograms were sprayed with sodium carbonate (10%).

Ammoniacal Silver (AS) was prepared by treating silver nitrate (10%) with sodium hydroxide (2N) until precipitation was complete. The mixture was treated with ammonia (0.88 sp.gr.) dropwise until a clear solution was first obtained.

Chlorimide reagents 2,6-Dichloroquinone chlorimide (DCQ) or 2,6-dibromoquinone chlorimide (DBQ) were used as a 0.2% ethanolic solution. The chromatograms were resprayed with sodium carbonate (10%) after the chlorimide reagent.

Sodium amminoprusside (SAP) was used as a 0.05% solution in 20% aqueous ethanol containing 1% magnesium chloride.

Ferric Chloride (FEC) was used as a methanolic solution (0.005%).

Ferric Chloride - Ferricyanide (FFCN) was prepared by mixing equal volumes of 0.03% aqueous solutions of ferric chloride and potassium ferricyanide immediately before use.

Ferric Chloride/Bathophenanthroline (FBP) was prepared by spraying the chromatogram with FEC and, after allowing to dry at room temperature for 10 minutes, respraying with bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) 0.1% in methanol.

Alkaline Tetrazolium (AT) was prepared by dissolving 2,3,5-triphenyl-tetrazolium chloride (0.1%) in ethanolic sodium hydroxide (0.5 N) immediately before use.

Brentamine Fast Red (BFR) and Brentamine Fast Blue (BFB) were used as 0.5% aqueous solution followed by spraying with sodium carbonate (10%).

2.2.2.8.3. Gas-Liquid Chromatographic Methods

Gas-liquid chromatography was carried out using a Perkin-Elmer F.11 gas chromatograph fitted with a flame ionisation detector. The detector response was measured using a Hitachi 152 recorder with a full scale deflection of one millivolt.

Initial experiments were carried out using a one metre metal column (O.D. 1/8") or a two metre glass column (O.D. $\frac{1}{4}$") packed with 2.5% SE-30 on Chromosorb G (80-100 mesh, acid-washed, DMCS-treated). Later experiments utilised either 3% OV-1 on Chromosorb G (80-100 mesh, acid-washed DMCS-treated) or 3% OV-17 on Chromosorb W(HP, 80-100 mesh). The gas pressures were adjusted to give optimal conditions as described in Section 4.

2.2.2.8.3.1. Direct examination of 4-aminobiphenyl and 4-acetamidobiphenyl and some potential metabolites by GLC

Solutions of 4-aminobiphenyl, 4-acetamidobiphenyl and various hydroxylated derivatives, and 4-nitrobiphenyl and 4-nitrosobiphenyl in ether were separately injected onto the gas chromatograph and the retention times and peak shapes at different column temperatures recorded.

2.2.2.8.3.2. Chromatography of acetyl derivatives

Solutions of 4-aminobiphenyl, 4-acetamidobiphenyl and various hydroxylated derivatives were prepared in pyridine and treated with acetic anhydride at 50° for up to three hours. Aliquots (2μl) were injected onto the gas chromatograph and the retention times and peak shapes recorded.

2.2.2.8.3.3. Chromatography of trifluoracetyl (TFA) derivatives

Solutions of 4-aminobiphenyl, 4-acetamidobiphenyl and various hydroxylated derivatives were prepared in pyridine and treated with trifluoroacetic anhydride at 50° Aliquots (2μl) were injected onto the

gas chromatograph and the retention times and peak shapes recorded.

2.2.2.8.3.4 Chromatography of methyl derivatives

Solutions of 4-aminobiphenyl, 4-acetamidobiphenyl and various hydroxylated derivatives were prepared in ether and treated with a solution of diazomethane. Aliquots of the reaction mixture were injected onto the gas chromatograph and the retention times and peak shapes recorded.

2.2.2.8.3.5. Chromatography of trimethylsilyl (TMS)

Solutions of 4-aminobiphenyl, 4-acetamidobiphenyl and various hydroxylated derivatives were prepared in acetonitrile and treated with various "silylating" agents. The silylating agents were prepared by dissolving various proportions of hexamethyldichlorosilane with trimethylchlorosilane in an organic basic solvent. Amongst the solvents tried were pyridine, pyrrolidine, thiethylamine and diethylamine. Latterly these were superceded by bis-silylacetamide or trimethylsilylimidazole.

2.2.2.8.3.6. Choice of Internal Standards

A wide variety of aromatic hydrocarbons were examined as internal standards as these were easily available pure, were easily extractable into organic solvents and were not affected by derivatizing reagents or mild oxidation conditions. Additionally, a number of 2-aminofluorenols and 2-aminobiphenylylols were studied because of their structural similarities to potential 4-aminobiphenyl metabolites. These potential standards were examined by GLC and the retention times recorded.

2.2.2.8.3.7. Conversion of 4-hydroxylaminobiphenyl to 4-nitrosobiphenyl

Solutions of 4-hydroxylaminobiphenyl were prepared in chloroform and treated with a variety of oxidising agents. Amongst the oxidants used were ferric chloride, potassium dichromate, nitric acid, perchloric acid and potassium ferricyanide. The organic phase was examined by GLC and the retention times of any products recorded.

2.2.2.8.3.8. Determination of ring hydroxylated products by GLC

Incubates (prepared as described in 2.2.2.2) which had been used to oxidise metabolically either 4-aminobiphenyl or 4-acetamidobiphenyl were transferred to screw-capped Sovirel tubes containing sodium chloride (1g) and internal standard. The incubates were extracted with methylene dichloride (3 x 5 ml) which was then concentrated under a stream of nitrogen at 55°. The residue was dissolved in acetonitrile and a granule of anhydrous calcium chloride added. Bis-silylacetamide was added and the mixture heated at 50° for twenty minutes in a stoppered tube. The peak heights of the trimethylsilyl derivatives of the hydroxylated compounds were determined relative to the peak height of the internal standard.

2.2.2.8.3.9 Determination of 4-hydroxylaminobiphenyl by GLC

Incubates containing 4-hydroxylaminobiphenyl were treated with internal standard and transferred to screw-capped Sovirel tubes containing sodium chloride (1g) and potassium ferricyanide (0.2 ml 10%). The mixture was extracted with freshly-distilled petroleum ether (bp 40-60°), 2 x 5 ml. The organic phase was concentrated by evaporation at 55°

under a stream of nitrogen. During the evaporation 20 μl of isopropanol was added to prevent volatilisation of 4-nitrosobiphenyl. The tubes were placed in crushed ice and stoppered, allowing the condensing vapour to wash the 4-nitrosobiphenyl and the internal standard into the collecting nipple. The peak height of the 4-nitrosobiphenyl relative to the peak height of the internal standard was determined.

2.2.2.8.4 High Performance Liquid Chromatography

High performance liquid chromatography was carried out using an apparatus consisting of dual pumps in conjunction with a model 660 solvent programmer and model 440 ultra-violet absorbance detector set at either 254 nm or 280 nm (Waters Co., Milford, Mass.).

The solvent programmer was set at curve 6 to give a linear solvent change. For the analysis of 4-aminobiphenyl and metabolites the initial solvent composition was phosphate buffer (0.03 M, pH 7.4) 65%; acetonitrile (U.V. grade, glass-distilled, Burdick and Jackson, Muskegon, Michigan) 35%. Solvents were filtered through a Millipore 5 micron solvent-proof disc prior to use. During the course of the run the solvent composition changed over three minutes to give a final composition of phosphate buffer 40% and acetonitrile 60%, the flow rate being maintained at 2 ml per minute.

For the analysis of 4-acetamidobiphenyl and metabolites the initial solvent composition was phosphate buffer 70%, acetonitrile 30%. This was changed during seven minutes using linear programme number six to

give a final composition of phosphate buffer 25%, acetonitrile 75%.

The column consisted of a stainless steel tube 20 cm long, having an internal diameter of 4 mm, packed with VYDAC TM201 reverse phase C-18 bonded to a silica support of 10 micron particle size (Applied Science Laboratories, State College, Pennsylvania). The equipment was fitted with a Waters U6K injection system.

2.2.8.4.1 Quantitative determination of hydroxylated metabolites of 4-aminobiphenyl and 4-acetamidobiphenyl using HPLC

Following the incubations with 4-aminobiphenyl or 4-acetamidobiphenyl (as described in 2.2.2.2.) the enzymic reaction was stopped by the addition of acetone (1 ml), and the metabolites were extracted into ethyl acetate (5 ml) using a rotary shaker for thirty minutes. The ethyl acetate was removed after the mixture had been centrifuged, and evaporated under a stream of nitrogen at 40°. The residue was dissolved in acetonitrile (0.5 ml), and an aliquot (10 μl) was injected onto the chromatograph. The metabolites were quantitated by use of factors obtained by carrying standard amounts of metabolites through the whole procedure.

2.2.2.9 Colorimetric Methods

2.2.2.9.1 Determination of 4-hydroxylaminobiphenyl using sodium amminoprusside (SAP)

Standard amounts of 4-hydroxylaminobiphenyl were added to microsomal suspensions which were immediately mixed and treated with ethanol to precipitate protein. The mixtures were centrifuged at 2000 rpm and aliquots were removed and treated with SAP reagent (SAP 0.25%

in 0.01% magnesium chloride). The absorbance maximum was determined, using a Unicam SP 800 spectrophotometer, after the colour was allowed to develop maximally (30 min). The absorbance equivalent to each concentration was determined.

2.2.2.9.2. Determination of 4-(N-hydroxyacetamido)-biphenyl using ferric chloride

Standard amounts of 4-(N-hydroxyacetamido)-biphenyl were added to a microsomal suspension which was then extracted with freshly-distilled diethyl ether. An aliquot of the extract was evaporated to dryness and redissolved in anhydrous methanol containing anhydrous ferric chloride (0.005%). The absorption maximum was determined using a Unicam SP 800 spectrophotometer and the absorbance equivalent to each concentration of hydroxamic acid was obtained.

2.2.2.9.3. Determination of 4-amino-3-hydroxybiphenyl by conversion to 1,7-diphenyl-4-amino-3H-isophenoxazin-3-one

Standard amounts of 4-amino-3-hydroxybiphenyl were added to microsomal suspensions which were then treated with trichloracetic acid. The mixtures were centifuged at 2000 rpm and aliquots of the supernatant were treated with potassium ferricyanide (20%) at room temperature with constant agitation for twenty minutes. The reaction mixtures were extracted with chloroform which was subsequently dried using sodium sulphate. The absorption maximum was determined using a Unicam SP 800 spectrophotometer. A concentration versus absorbance relationship was established by determining the absorbance equivalent to each concentration of 4-amino-3-hydroxybiphenyl added to the microsomes.

2.3. RESULTS AND DISCUSSION

2.3.1 Paper Chromatography

The reverse phase technique of Gasparic et al. (1955) for the separation of hydroxylated derivatives of 4-aminobiphenyl or 4-acetamidobiphenyl gave very poor results. In agreement with Bradshaw (1959) it was found that the substances gave irreproducible results varying from run to run and often streaked the whole length of the paper. Variation of solvent or of temperature or of support paper failed to stabilise the system and it was therefore abandoned.

2.3.2 Thin Layer Chromatography (T L C)

Thin layer chromatography has been widely used for the separation of aromatic amines and their metabolites (Smith, 1978, and references therein) and was used by Booth and Boyland (1964) in their study of 4-aminobiphenyl metabolism. In agreement with these authors it was found that the technique was quick, reproducible and when combined with specific detection reagents allowed the recognition of individual hydroxylated metabolites. The chromatographic properties of 4-aminobiphenyl and 4-acetamidobiphenyl together with some potential metabolites are recorded in Table 2.3.2 and their responses to various detection reagents are recorded in Table 2.3.3.

Compounds with free amino groups could readily be detected by diazotisation followed by coupling with an alkaline phenol. Of the phenols

tested for this purpose, 1- or 2- naphthol produced the best response. This reagent was not suitable for 4-amino-3-hydroxybiphenyl as diazotisation produced an internal diazo oxide which did not couple with alkaline phenols, but generally produced a green to yellow colour with the alkali alone. This substance could be recognised by the black coloration produced with ammoniacal silver nitrate, which formed faster than with the other aminophenols but slower than with the hydroxylamine. 4-Hydroxylaminobiphenyl was easily detected by its specific response to SAP, FBP and AT. Compounds with free amino groups also gave an immediate reaction with either DAB or DAC; these reagents also gave positive reactions with acetamido and nitro compounds on being allowed to stand for several hours. In these cases the colours produced were of the parent amine, presumably being produced by hydrolysis and reduction respectively. Phenolic compounds derived from either 4-aminobiphenyl or 4-acetamidobiphenyl could also be detected by their response to a range of diazonium compounds or chlorimide reagents. The hydroxamic acid formed from 4-acetamidobiphenyl can readily be recognised by the characteristic colour produced in the presence of ferric ions.

Amongst the solvent systems examined S 1 was the best for separating the hydroxylated derivatives of 4-aminobiphenyl whilst S 4 was the best for 4-acetamido derivatives.

It should be noted that no detection reagents were found which gave a response with either N-(4-biphenylyl)-glycolamide or N-(4-biphenylyl)-

oxamic acid. However, the latter compound was unlikely to be formed in microsomal incubation systems.

2.3.3. Gas-liquid Chromatography (GLC)

The examination of the chromatographic properties of 4-aminobiphenyl and 4-acetamidobiphenyl in their derivatives revealed that whilst the parent compounds gave sharp symmetrical peaks using a number of different columns, the hydroxylated derivatives, that is the potential metabolites, did not. Generally the aminophenols gave very broad peaks and required a large amount of substance to be detected. The acetamidophenols also gave broad peaks with long retention times. The N-hydroxy derivatives of either 4-aminobiphenyl were unstable on all columns used; the arylhydroxylamine gave rise to multiple peaks having chromatographic properties identical to 4-aminobiphenyl, 4-nitrosobiphenyl and 4-nitrobiphenyl. The hydroxamic acid gave products which were identical to 4-acetamidobiphenyl, 4-nitrosobiphenyl and 4-aminobiphenyl when a metal column was used. When a glass column was used the principal product was identical with 4-acetamido-3-hydroxybiphenyl.

Attempts at stabilisation or increasing the volatility of these compounds by use of derivatives was also associated with problems. The N,O-diacetyl derivatives of ring hydroxy derivatives of 4-aminobiphenyl were stable and gave good peaks on the gas chromatograph; however, the N,O-diacetyl derivative of 4-hydroxylaminobiphenyl (which is the same compound as that produced by the acetylation of 4-acetamido-N-hydroxybiphenyl) was converted to the 3-acetoxy compound during GLC. The reactivity of the N,O-diacyl moiety appeared even greater when trifluoretic anhydride

Table 2.3.1 Composition of solvent systems used in conjunction with silica gel G plates for TLC

S1	Petroleum Ether (bp 60°-80°): Acetone	7:3
S2	Chloroform: Methanol: Ammonia (SG 0.88)	95:5:1
S3	Chloroform: Methanol: Water	95:10:1
S4	Cyclohexane: t-Butanol: Acetic acid: Water	16:4:2:1
S5	Carbon Tetrachloride: Ethyl Acetate: Dichloromethane: Formic Acid	35:25:25:5
S6	Benzene: Methanol	9:1
S7	Benzene: Dioxane: Diethylamine: Water: Methanol	70:17.5:7.5:1:4
S8	Cyclohexane: Ethyl Acetate	7:3
S9	Benzene: Methanol: Acetic Acid	45:8:4
S10	Chloroform: Ethyl Acetate: Acetic Acid: Water	12:6:2:1

Table 2.3.2 Chromatographic properties (R_{fx} 100) of 4-aminobiphenyl, 4-acetamidobiphenyl and some possible metabolites during thin layer chromatography

COMPOUND \ SYSTEM*	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
4-aminobiphenyl	55	89	73	56	22	73	75	52	72	94
4-amino-3-hydroxybiphenyl	42	56	73	30	9	49	57	27	64	66
4-amino-2'-hydroxybiphenyl	34	65	61	25	9	48	63	25	62	71
4-amino-4'-hydroxybiphenyl	28	55	72	14	5	40	56	21	55	66
4-hydroxylaminobiphenyl	78	86	95	81	86	88	86	36	77	98
4-nitrosobiphenyl	86	94	96	84	86	88	86	89	90	98
4-acetamidobiphenyl	38	76	84	54	62	51	63	21	72	86
4-acetamido-3-hydroxybiphenyl	36	58	78	47	60	47	28	20	67	84
4-acetamido-2'-hydroxybiphenyl	21	43	63	34	49	31	41	8	61	76
4-acetamido-4'-hydroxybiphenyl	19	40	61	25	49	27	37	7	56	77
4-acetamido-N-hydroxybiphenyl	36	50	86	62	46	56	54	19	77	81
N-(4-biphenylyl)-glycolamide	24	52	76	47	46	44	48	9	69	76
N-(4-biphenylyl)-oxamic acid	0	0	0	4	4	0	8	0	0	5
4-nitrobiphenyl	82	92	95	89	92	86	86	87	90	96

*Composition of solvent system described in Table 2.3.1

Table 2.3.3 Response to detection reagents of 4-aminobiphenyl, 4-acetamidobiphenyl and some possible metabolites

COMPOUND \ REAGENT[1]	UV-254	UV-366	1 - N	2 - N	4-HB	2 - HB	HR	DAB	DAC	DNA
4-amino-biphenyl	blue	grey	red	red	orange	red	orange	yellow	purple	yellow
4-amino-3-hydroxybiphenyl	blue	orange	green	yellow	green	yellow	grey	gold	purple	orange
4-amino-2'-hydroxybiphenyl	blue	orange	red	red	brown	orange	orange	gold	purple	orange
4-amino-4'-hydroxybiphenyl	blue	orange	purple	red	green	brown	brown	gold	purple	brown
4-hydroxylaminobiphenyl	blue	blue	brown	brown	yellow	brown	orange	gold	purple	NR
4-nitrosobiphenyl	dark	purple	NR[2]	NR	NR	NR	NR	yellow	purple	NR
4-acetamidobiphenyl	dark	dark	NR	NR	NR	NR	NR*	NR*	NR*[3]	NR
4-acetamido-3-hydroxybiphenyl	blue	blue	NR	NR	NR	NR	NR	NR*	NR*	red
4-acetamido-2'-hydroxybiphenyl	blue	orange	NR	NR	NR	NR	NR	NR*	NR	orange
4-acetamido-4'-hydroxybiphenyl	blue	orange	NR	NR	NR	NR	NR	NR*	NR*	red
4-acetamido-N-hydroxybiphenyl	blue	blue	NR	NR	NR	NR	NR	NR*	NR*	NR
N-(4-biphenylyl)-glycolamide	blue	dark	NR	NR	NR	NR	NR	NR	NR	NR
N-(4-biphenylyl)-oxamic acid	blue	dark	NR	NR	NR	NR	NR	NR	NR	NR
4-nitrobiphenyl	dark	dark	NR	NR	NR	NR	NR	NR*	NR*	NR

1. Composition of detection reagents described in text, Section 2.2.2.8.2.2.
2. NR indicates no response to detection reagents.
3. Compounds marked * give the same response as the deacetylated compound after leaving for 24 hours.

Table 2.3.3 Continued

COMPOUND \ REAGENT[1]	NNCD	AS	DCQ	DBQ	SAP	FEC	FFCN	FBP	AT	BFR
4-aminobiphenyl	yellow	grey	brown	orange	red	NR	NR	NR	NR	yellow
4-amino-3-hydroxybiphenyl	orange	black	blue	green	yellow	brown	blue	NR	NR	rose
4-amino-2'-hydroxybiphenyl	red	grey	blue	blue	orange	brown	orange	NR	NR	orange
4-amino-4'-hydroxybiphenyl	yellow	grey	grey	grey	grey	violet	purple	NR	NR	yellow
4-hydroxylaminobiphenyl	orange	black	green	green	purple	yellow	blue	red	red	yellow
4-nitrosobiphenyl	orange	NR	NR	NR	purple	NR	NR	NR	NR	NR
4-acetamidobiphenyl	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
4-acetamido-3-hydroxybiphenyl	orange	grey	magenta	violet	NR	NR	blue	NR	NR	rose
4-acetamido-2'-hydroxybiphenyl	red	grey	blue	blue	NR	NR	purple	NR	NR	pink
4-acetamido-4'-hydroxybiphenyl	red	grey	grey	grey	NR	pink	blue	NR	NR	pink
4-acetamido-N-hydroxybiphenyl	NR	grey	green	brown	NR	pink	mauve	orange	NR	NR
N-(4-biphenylyl)-glycolamide	green	NR	NR	NR	NR	NR	NR	NR	NR	NR
N-(4-biphenylyl)-oxamic acid	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
4-nitrobiphenyl	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

1. composition of detection reagents as described in text, Section 2.2.2.8.2.
2. NR indicates no response to detection reagents.

was used as an acylating agent, as in this case the product isolated from the reaction was identical in m.p. and chromatographic properties with that isolated from the acylation of 4-amino-3-hydroxybiphenyl.. Examination of the infra-red spectra of these compounds showed they were identical and possessed absorption bands at 1300 and 3400 nm, indicative of a secondary amide.

A similar rearrangement and breakdown occurred during the chromatography of the products obtained by treating 4-hydroxylaminobiphenyl with either diazomethane or "silylating" agents. The retention times of 4-aminobiphenyl and some related compounds and derivatives using an SE 30 column are recorded in Table 2.3.4.

From this data it became clear that if the hydroxylamine could be quantitatively converted to 4-nitrosobiphenyl a method of assay might be developed. 4-Nitrosobiphenyl gives a peak on the chromatograph which is well separated from the solvent front, the parent amine and 4-nitrobiphenyl. A number of oxidising agents were examined, but under conditions which may be useful for a routine assay they converted the hydroxylamine into 4-nitrosobiphenyl together with 4-nitro- and 4-aminobiphenyl. The successful conversion of 4-hydroxylaminobiphenyl to 4- nitrosobiphenyl, without the formation of the other compounds, was achieved by the use of potassium ferricyanide as oxidising agent under slightly acid conditions. A number of compounds were examined as potential internal standards (Table 2.3.5) and from the results it was found that

Table 2.3.4. Gas chromatographic properties (R_T) of 4-aminobiphenyl, 4-acetamidobiphenyl and some derivatives together with some potential internal standards[1]

Compound	parent compound	derivative			
		acetyl	methyl	TFA	TMS
4-aminobiphenyl	50[2]	170	60	60	60
4-nitrosobiphenyl	38	NC[3]	NC	NC	NC
4-hydroxylaminobiphenyl	38, 50, 80	170,300	75, 100, 220	145	38, 60, 120
4-amino-3-hydroxybiphenyl	110	300	100	145	120
4-amino-4'-hydroxybiphenyl	130	690	170	180	190
4-acetamidobiphenyl	170				
4-acetamido-3-hydroxybiphenyl	(ND 15)[4]				
4-acetamido-4'-hydroxybiphenyl	(ND 20)				
4-acetamido-N-hydroxybiphenyl	38, 50, 170				
4-nitrobiphenyl	80				
biphenyl	24				
fluorene	85				
naphthalene	15				
anthracene	69				
phenanthrene	68				
3-methylphenanthrene	93				

1 using a 2m column comprising 2.5% SE 30 on chromosorb G (AW, DMCS-treated) at 200°.
2 results are given in seconds.
3 N.C. indicates no change in R_T in presence of derivatizing agent
4 N.D. indicates nothing detected at time indicated in minutes.

Table 2.3.5 Gas chromatographic properties (R_T) of 4-aminobiphenyl, 4-acetamidobiphenyl, some derivatives and some potential internal standards

	3% OV1 on Chromosorb G'		3% OV17 on Chromosorb G'	
	parent compound	TMS derivative	parent compound	TMS derivative
4-aminobiphenyl	3.25	3.55	2.45	3.0
4-amino-3-hydroxybiphenyl	7.50	4.55	5.50	3.45
4-amino-2'-hydroxybiphenyl	5.25	4.55	4.10	3.55
4-amino-4'-hydroxybiphenyl	ND(20)[2]	10.0	7.50	7.35
4-hydroxylaminobiphenyl	2.05; 3.25	NC[3]	2.0; 2.45	NC
4-nitrosobi phenyl	2.05	NC	2.0	NC
4-nitrobiphenyl	4. 20	NC	5.10	NC
N-methyl-4-aminobiphenyl	-	-	3.20	-
3-methylphenanthrene	-	-	3.30	NC
4-acetamidobiphenyl	-	4.35	9.30	3.20
4-acetamido-3-hydroxybiphenyl	-	6.15	3.30	4.55
4-acetamido-2'-hydroxybiphenyl	-	5.30	16.0	4.55
4-acetamido-4'-hydroxybiphenyl	-	11.35	ND(30)	8.55
4-acetamido-N-hydroxybiphenyl	-	6.15	3.30, 9.30	4.55
4-(biphenylyl)-glycolamide	-	ND(30)	ND(30)	14.0
4-(biphenylyl)-oxamic acid	-	ND(30)	ND(30)	ND(30)
2-amino-1-fluorenol	-	9	-	8.10
2-amino-3-fluorenol	-	9.20	-	7.10

[1]Column details are given in text Section 2.2.2.8.3

[2] ND indicated that nothing was detected during the time indicated in min.

[3] NC indicates that no change was observed after treatment with silylating agents.

either 3-methylphenanthrene or N-methyl-4-aminobiphenyl were suitable. The separation of 4-nitrosobiphenyl from 4-aminobiphenyl and the internal standards is shown in Figure 2.3.1. Using this system it was possible to construct a calibration curve which was linear over the range 50 to 500 nano moles and which enabled the N-hydroxylation of 4-aminobiphenyl carried out by microsomal preparations to be studied quantitatively.

The determination of the aminophenols and the hydroxylated products from 4-acetamidobiphenyl proved to be far more difficult and required that the metabolites be convertedto their trimethylsilyl derivatives. A number of "silylating" agents were tried under different conditions of solvent, basicity and reaction temperature.

All the hydoxylated substances examined were converted to trimethyl-silyl derivatives by heating the substance with bis-silyl acetamide at 50° for twenty minutes in acetonitrile. The properties of these derivatives on OV1 and OV 17 columns are recorded in Table 2.3.5.

Unfortunately, whilst these derivatives had good peak shapes and convenient retention times it was very difficult to differentiate between the 3-hydroxy and 2'-hydroxy compound derived from either 4-aminobiphenyl or 4-acetamidobiphenyl. Furthermore, the N-hydroxy derivative could only be quantitated as the rearranged 3-hydroxy compound, clearly undesirable when the possibility existed that metabolic hydroxylation could occur at both sites of the molecule. 4-(Biphenylyl)-glycolamide gave only a very poor response under the conditions used. 2-Amino-3-fluorenol (which is commercially available) was easily silylated and is a potentially useful internal standard.

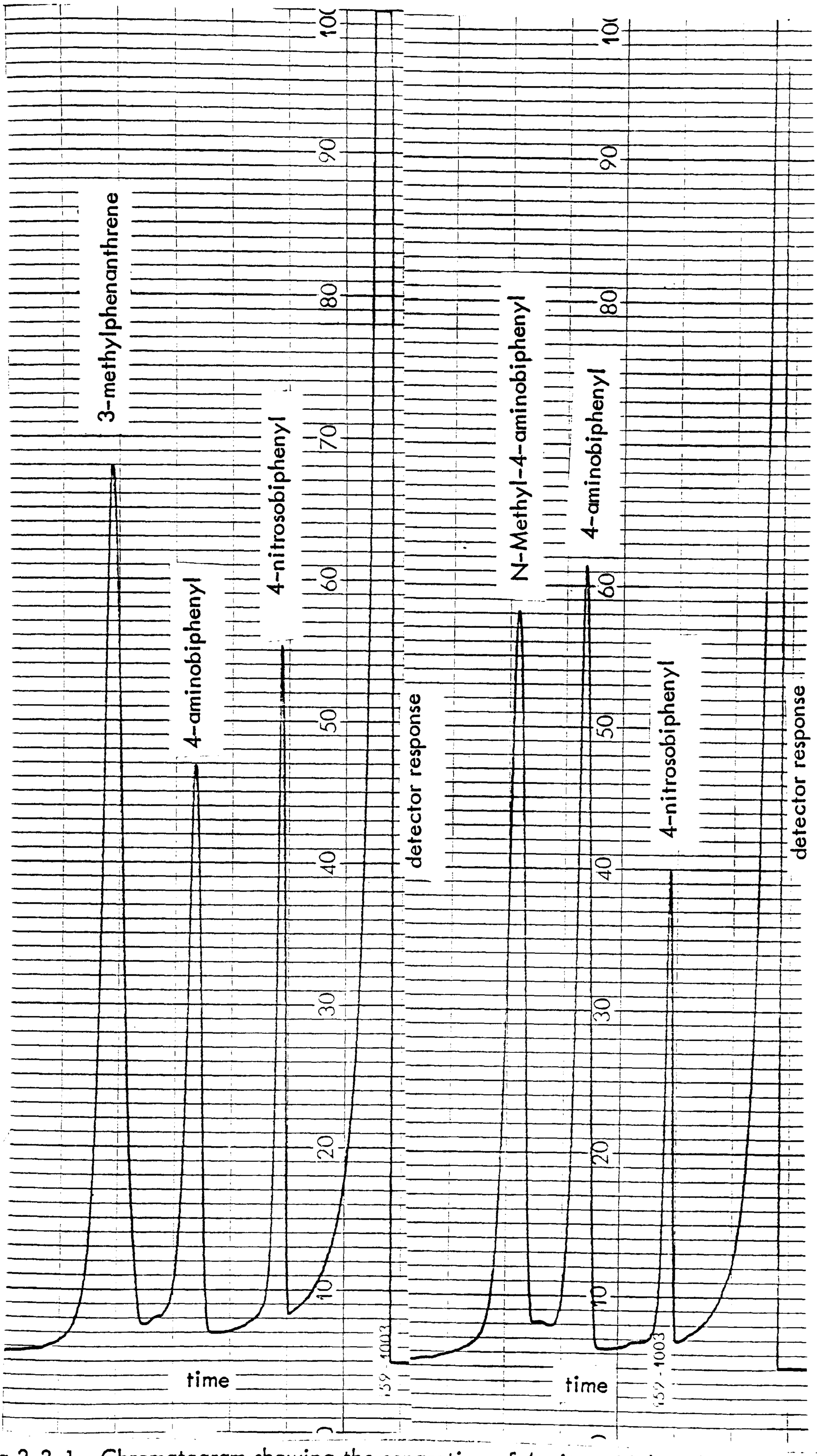


Fig 2.3.1 Chromatogram showing the separation of 4-nitrosobiphenyl from 4-aminobiphenyl and internal standards

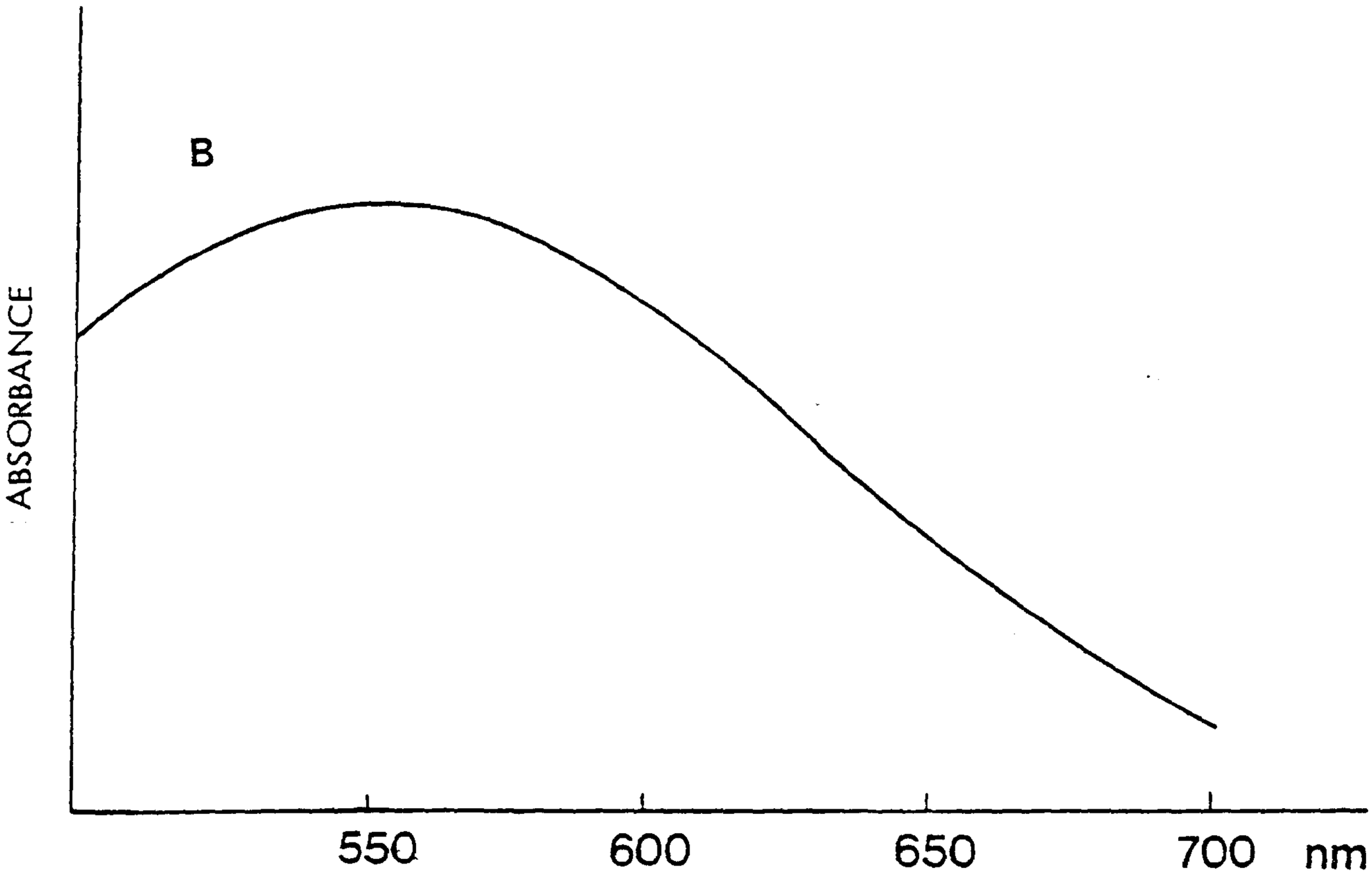
A further complication arose in the fact that tissue preparations contained material which gave interfering peaks on the chromatograph.

It is clear that GLC does not lend itself to the simultaneous determination of the products of hydroxylation at a number of sites on 4-aminobiphenyl and 4-acteamidobiphenyl. The number of samples to be analysed would make the development of a specific assay for each specific site of oxidation far too tedious and time-consuming. However, the techniques which were developed here were of considerable use in conjunction with TLC in confirming the identity of substances, following indications by R_f values and response to detection reagents.

2.3.4 Colorimetric Methods

The reaction of arylhydroxylamines with sodium amminoprusside to form coloured complexes reported by Boyland and Nery (1964) has been confirmed in this study with 4-hydroxylaminobiphenyl. The absorption spectrum and absorbance vs. concentration graph are shown in Figure 2.3.2. The results obtained in aqueous solution indicate the sensitivity of the reaction; however, when the reaction was carried out in material derived from microsomal incubates the sensitivity was reduced by a factor of ten.

The formation of a coloured complex between 4-acetamido-N-hydroxybiphenyl and ferric ions, which was used as a quantitative method by Booth and Boyland (1964), was also confirmed. Because of the need to carry out an extraction and yet gain information on only one site



B. Absorption spectra of complex formed between 4-hydroxylaminobiphenyl and sodiumamminoprusside

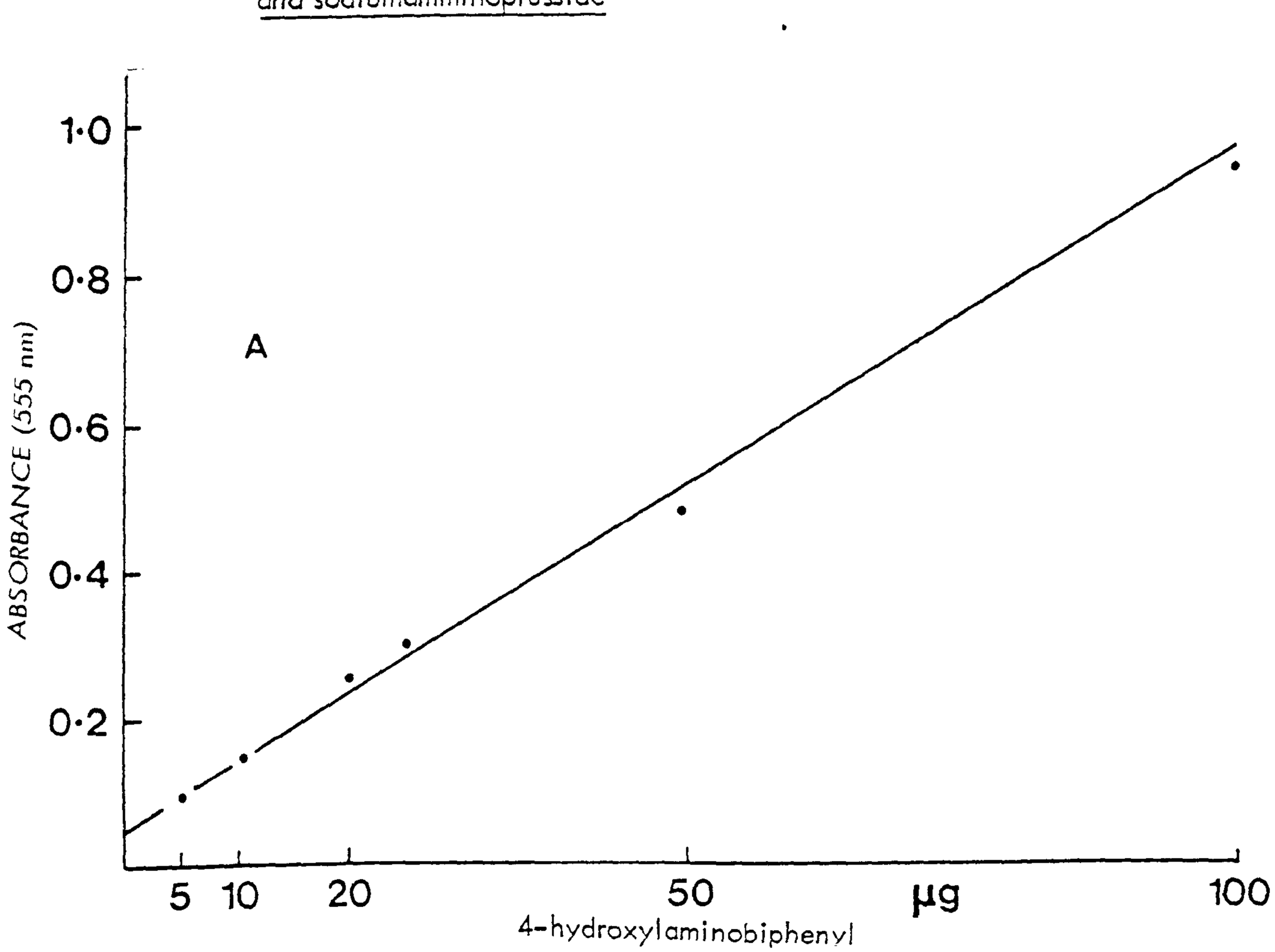
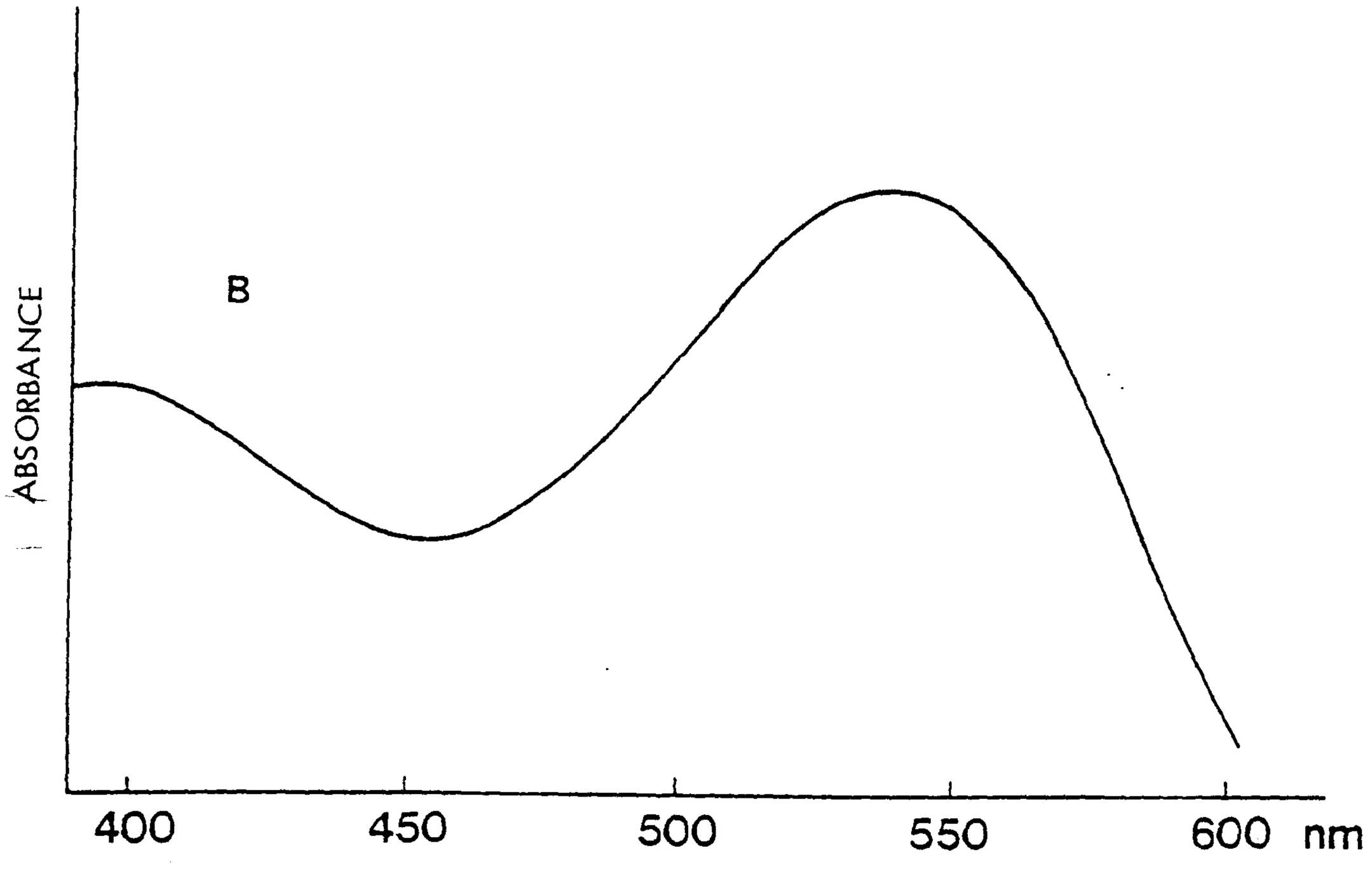


Fig. 2.3.2. A. Concentration curve of 4-hydroxylaminobiphenyl determined as its complex with sodium amminoprusside



B. Absorption spectra of the oxidation product of 4-amino-3-hydroxybiphenyl

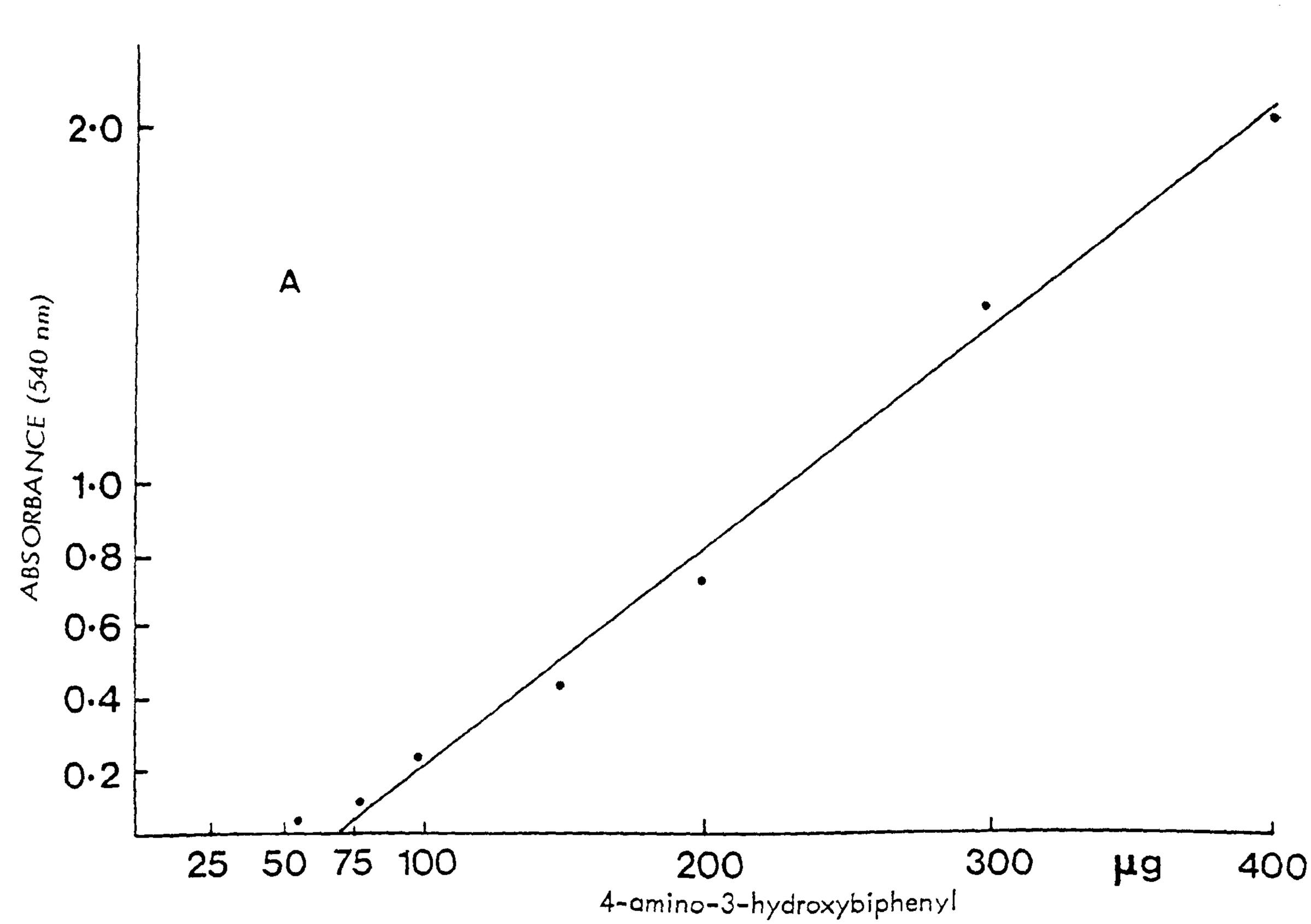


Fig. 2.3.3.A Concentration curve of oxidation product of 4-amino-3-hydroxybiphenyl

of oxidation, together with the risk of phenols being formed which may also give a positive reaction with ferric ions, the method was not used further.

The oxidation of 4-amino-3-hydroxybiphenyl to 1,7-diphenyl-4-amino-3H-isophenoxazin-3-one has previously been demonstrated by Nagasawa, Gutmann and Morgan (1959). As materials with structures of this type are often highly coloured it seemed that this reaction may be developed into a useful assay procedure for the determination of 4-amino-3-hydroxybiphenyl. The results obtained are shown in Figure 2.3.3; they show that whilst a coloured product is formed the low absorption of the product in the presence of microsomal constituents precluded this being developed into a useful assay.

2.3.5 High Pressure Liquid Chromatography (HPLC)

4-Aminobiphenyl, together with the possible metabolites discussed earlier, were examined by HPLC. The compounds gave characteristic peaks which were easily separated from each other by variation in the solvent composition. The R_T values, which are recorded in Table 2.3.6, were reproducible under the solvent gradient systems developed, and they allowed the establishment of an assay method which not only permitted the simultaneous determination of four sites of microsomal oxidation of 4-aminobiphenyl but which also enabled the further oxidation of 4-hydroxy-laminobiphenyl to either 4-nitroso- or 4-nitrobiphenyl to be detected.

The print-out for the standard compounds related to 4-aminobiphenyl is shown in Figure 2.3.4.

Table 2.3.6. The chromatographic properties of 4-aminobiphenyl, 4-acetamindibiphenyl and some metabolites and some related compounds using a HPLC system [1]

Compound	R_T (sec)
4-aminobiphenyl	300
4-amino-3-hydroxybiphenyl	175
4-amino-2'-hydroxybiphenyl	150
4-amino-4'-hydroxybiphenyl	120
4-N-hydroxylaminobiphenyl	225
4-nitrosobiphenyl	415
4-nitrobiphenyl	480
4,4-azobiphenyl	720
4,4-azoxybiphenyl	560
4-acetamidobiphenyl	335
4-acetamido-3-hydroxybiphenyl	255
4-acetamido-2'-hydroxybiphenyl	165
4-acetamido-4'-hydroxybiphenyl	122
4-acetamido-N-hydroxybiphenyl	500
4-N-(biphenylyl)-glycolamide	270
4-N-(biphenylyl)-oxamic acid	125

[1] The details of the chromatographic systems used are described in the text (Section 2.2.2.8.4).

Similar experience was obtained with 4-acetamidobiphenyl, although in this case the potential metabolites required a greater variation of solvent composition (see Section 2.2.2.8.4.1). The chromatogram (Figure 2.3.7) shows that oxidation on the 4'-, 2'- and 3- carbon atoms, and on the CH_3 group of the acetamido function, can readily be detected, and the metabolites so formed can be distinguished from each other and from the parent amide and the hydroxamic acid.

A single extraction of a microsomal incubate therefore allowed analysis of all the available potential hydroxylated metabolites of 4-aminobiphenyl, or 4-acetamidobiphenyl, respectively.

2.3.6 Metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl

The results obtained from the qualitative studies have supported the early observations of Booth and Boyland (1964) in that, using rabbit tissue, the principal sites of microsomal oxidation of 4-aminobiphenyl were found to be either the nitrogen atom or the adjacent 3- position in the nucleus, whereas oxidation of 4-acetamido biphenyl occurred principally at the 4'- position. These observations have now been extended to rats, mice, hamsters and dogs. In the case of the amine two minor pathways of metabolism were detected using thin layer chromatography.

These metabolites were first detected using tissue preparations from the guinea pig, a species which has a relatively low ability to hydroxylate 4-aminobiphenyl in the 3- position. The technique used was to incubate six flasks, each containing 5 μmole of substrate, for thirty minutes, then

extract the incubation mixture with diethyl ether as described earlier and bulk the ether extracts prior to examination by TLC. The areas of the chromatograms which corresponded in Rf to authentic compounds were removed from the supporting plate and eluted with methanol, and this was again concentrated and an aliquot was re-examined by TLC.

The remainder of the methanolic extract was evaporated to dryness, treated with bis-silylacetamide in acetonitrile and examined by GLC. The finding of metabolites which, as compared with authentic compounds, had identical chromatographic properties in several solvent systems and gave the same responses to detection reagents, and which gave silyl derivatives with identical properties to those prepared from the authentic compounds, was considered very good evidence for the formation of both 4-amino-4'-hydroxybiphenyl and 4-amino-2'-hydroxybiphenyl. These experiments were repeated using the above technique with hepatic microsomal preparations from rabbit, rats, mice and hamsters. In every case small amounts of the two new metabolites were detected. Using this technique, i.e. a combination of TLC and GLC, it was possible to find materials present on the TLC plates which had the properties of 4-nitrobiphenyl and 4-nitrosobiphenyl respectively. However, it is difficult to say whether 4-nitrobiphenyl was formed as a metabolite of 4-aminobiphenyl or whether it was caused by the on-column breakdown of either 4-hydroxylaminobiphenyl or 4-nitroso-biphenyl. It is known that the former compound does break down to a number of substances when injected onto GLC systems (see earlier discussion in

this Section); however, the nitroso compound gave only one peak. It may have been that the microsomal extracts broke down during TLC due to the presence of ether-soluble material, perhaps peroxides formed during the incubation procedure.

The metabolism of 4-acetamidobiphenyl was also examined using the above techniques, and again in agreement with the observations of Booth and Boyland (1964) it was found that hydroxylation at the 4'-position was the major route of metabolism.

Also in agreement with Booth and Boyland (1964) it was found that 4-acetamidobiphenyl was hydroxylated at the nitrogen atom but not at the 3- position in the nucleus. During metabolic studies with this compound a new minor metabolite was found which had the properties of 4-acetamido-2'-hydroxybiphenyl. Attempts at detecting N-(4-biphenylyl)-glycolamide, which would have been expected to have been formed as a metabolite (Fries, Kiese and Lenk, 1973) were unsuccessful. This may have been due to the lack of sensitivity of the methods used, as this material was detected using the HPLC system.

The quantitative metabolism of 4-aminobiphenyl and 4-acetamido-biphenyl in various species was studied using the assay based on HPLC described earlier. The chromatograms of extracts of typical incubation mixtures containing 4-aminobiphenyl as substrate and rabbit or hamster hepatic microsomes as enzyme source are shown in Figures 2.3.5 and 2.3.6; and those obtained using 4-acetamidobiphenyl as substrate are shown in

Figures 2.3.8 and 2.3.9. In these chromatograms the substances found in the qualitative study could be detected, together with N-(4-biphenyl)-glycolamide from 4-acetamidobiphenyl. Again a substance was formed from 4-amino-biphenyl which had the properties of 4-nitrobiphenyl. If the occurrence of this process - that is, the oxidation of an aromatic amine to an aromatic nitro compound - is substantiated then this will apparently be the first report of such a pathway in a mammalian system, although the process is known to occur in certain microbial systems.

In order to gain further information about the enzymes involved in the metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl a number of experiments were carried out establishing conditions for the comparative experiments using different species.

The results obtained by varying the incubation time are shown in Table 2.3.7 and Figure 2.3.10. Production of all the metabolites from 4-aminobiphenyl increased with time up to twenty minutes of incubation. A similar result was obtained when 4-acetamido biphenyl was used as substrate, except that no change in the level of the 2'-hydroxy compound was found after 5 minutes' incubation. A separate experiment was also carried out in which 4-aminobiphenyl was metabolised in the presence of fluoride ions. This was performed in order to establish whether fluoride had any inhibiting effect on microsomal oxidation, as it was routinely added to incubates in which 4-acetamidobiphenyl was a substrate. This was necessary in order to prevent deacetylation (see Section 3). The results show that no significant

a = 4-amino-4'-hydroxybiphenyl
b = 4-amino-2'-hydroxybiphenyl
c = 4-amino-3-hydroxybiphenyl
d = 4-hydroxylaminobiphenyl
e = 4-aminobiphenyl
f = 4-nitrosobiphenyl
g = 4-nitrobiphenyl

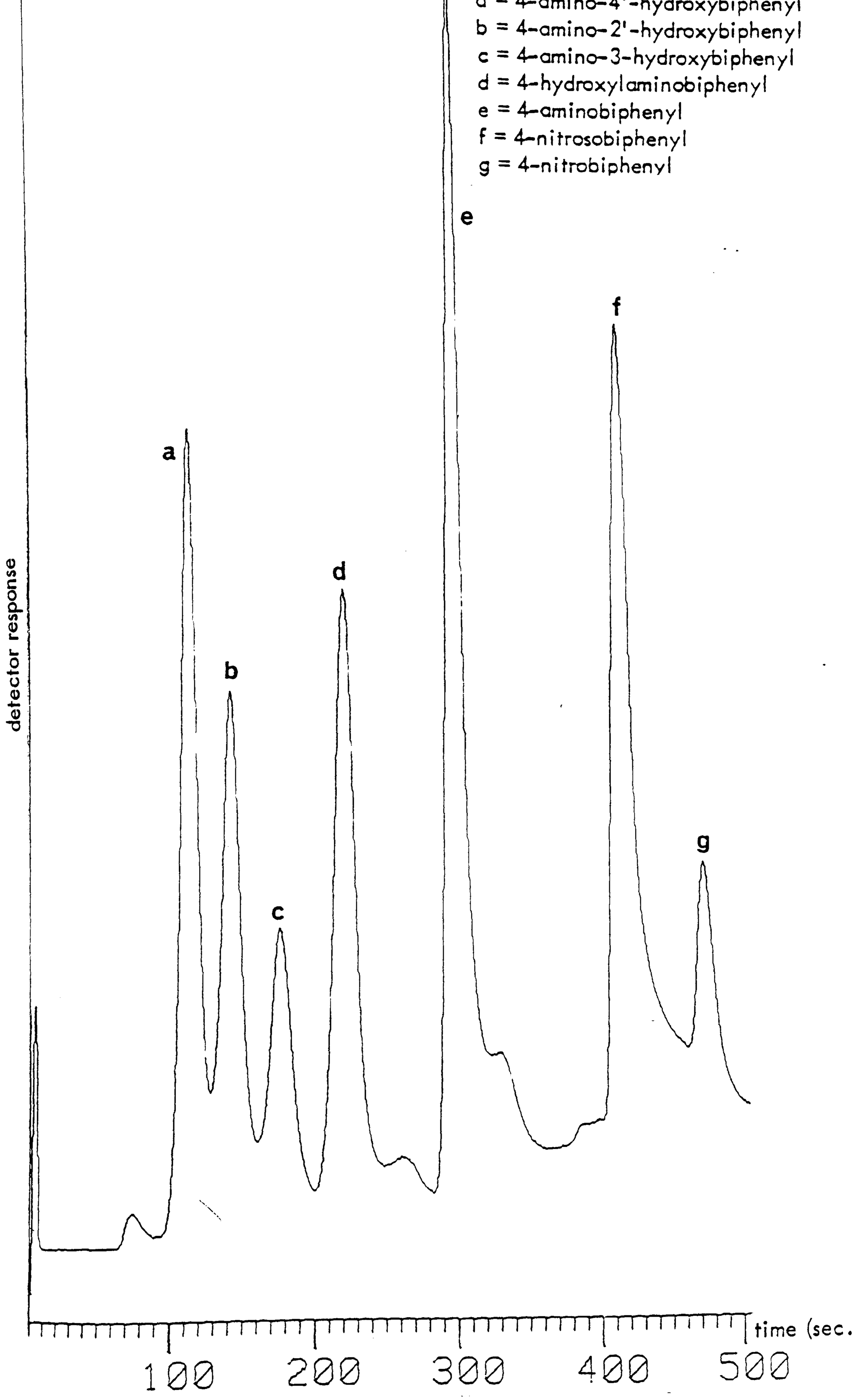


Fig 2.3.4. Computer printout showing standard compounds related to 4-aminobiphenyl separated by HPLC

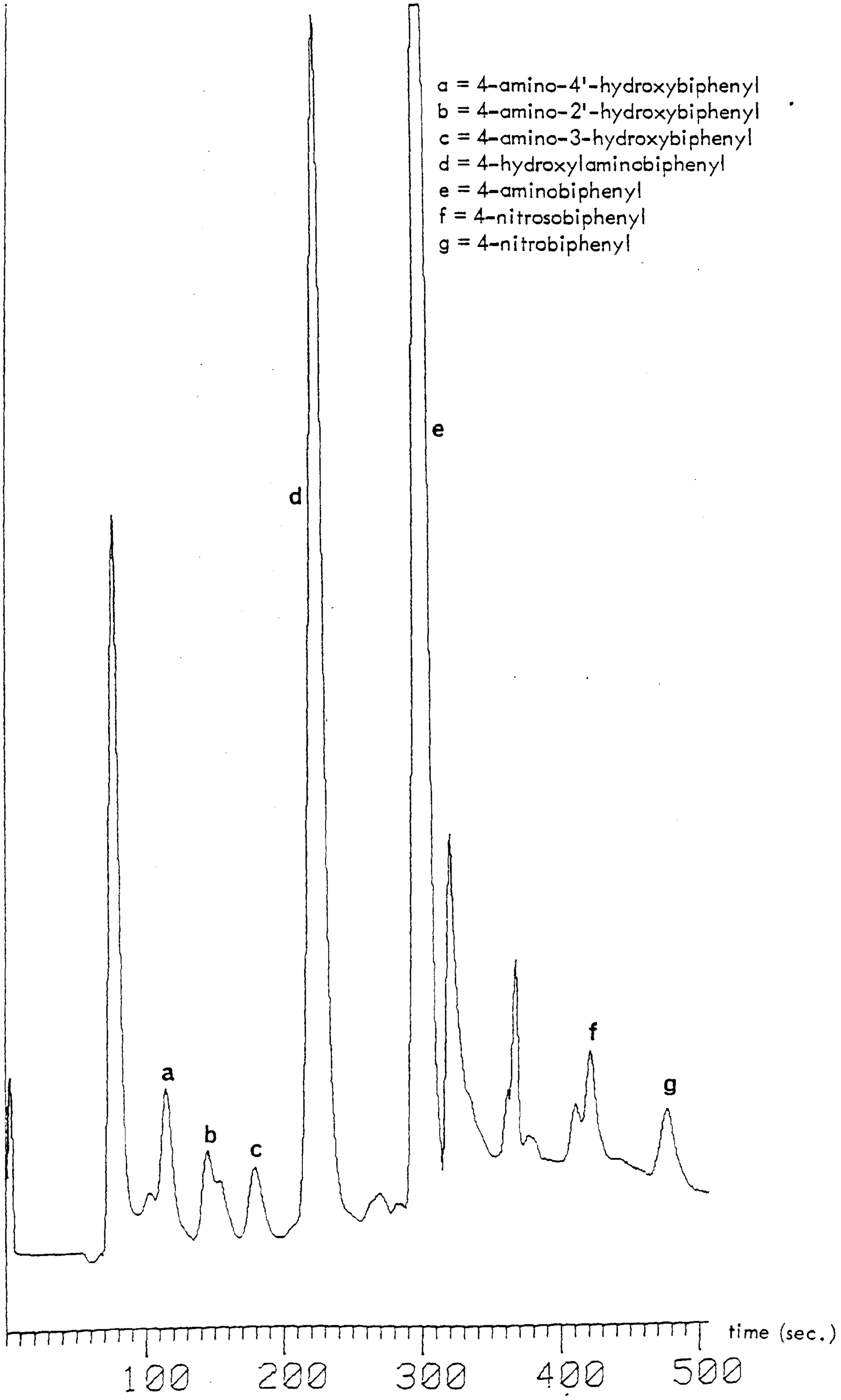


Fig. 2.3.5. Chromatogram of extract from a microsomal incubate of 4-aminobiphenyl using rabbit hepatic tissue

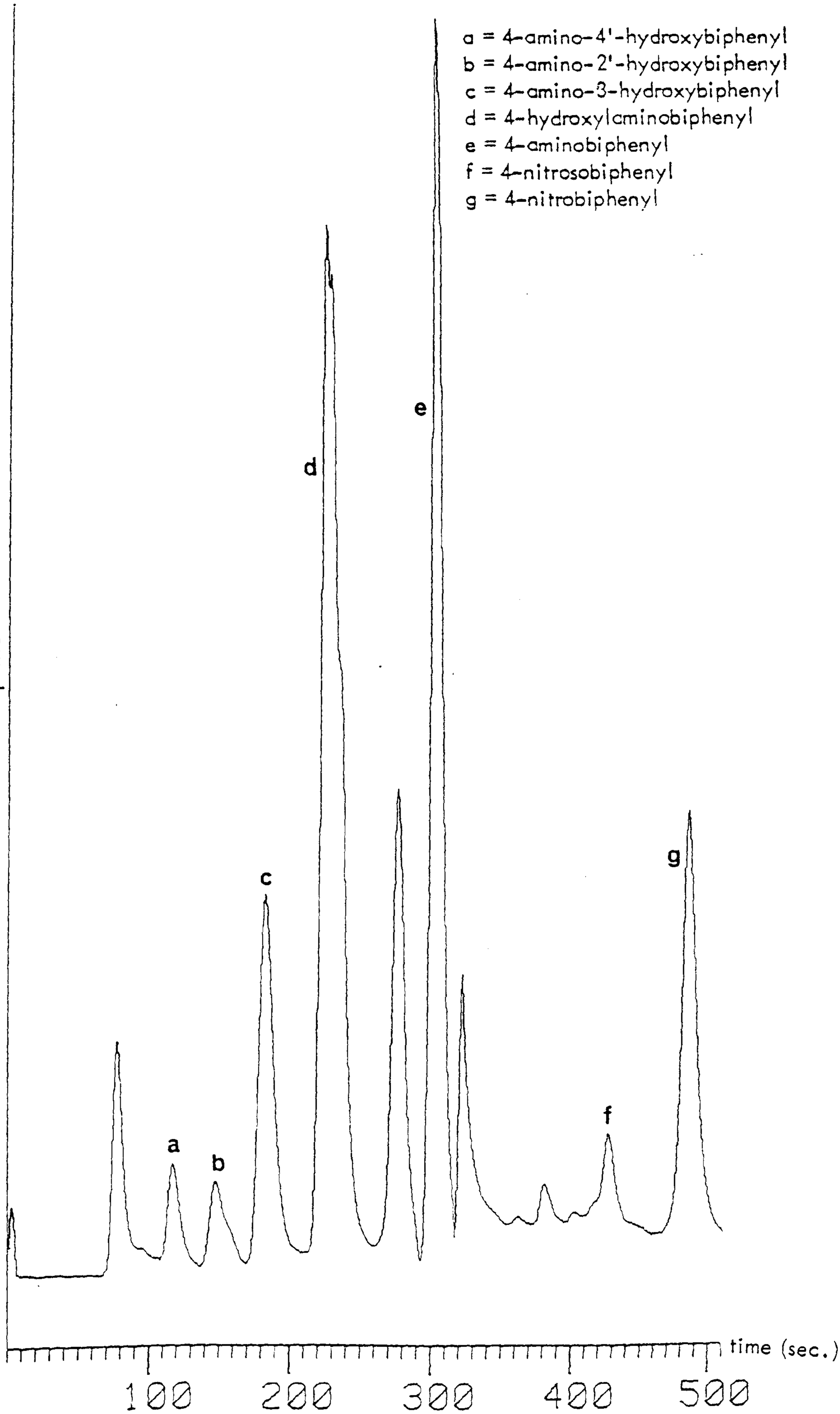


Fig. 2.3.6. Chromatogram of extract from a microsomal incubate of 4-aminobiphenyl using hamster hepatic tissue

a = 4-acetamido-4'-hydroxybi phenyl
b = 4-acetamido-2'-hydroxybiphenyl
c = 4-acetamido-3-hydroxybiphenyl
d = 4-N-(biphenylyl)glycolamide
e = 4-acetamidobiphenyl
f = 4-aminobiphenyl
g = 4-acetamido-N-hydroxybiphenyl

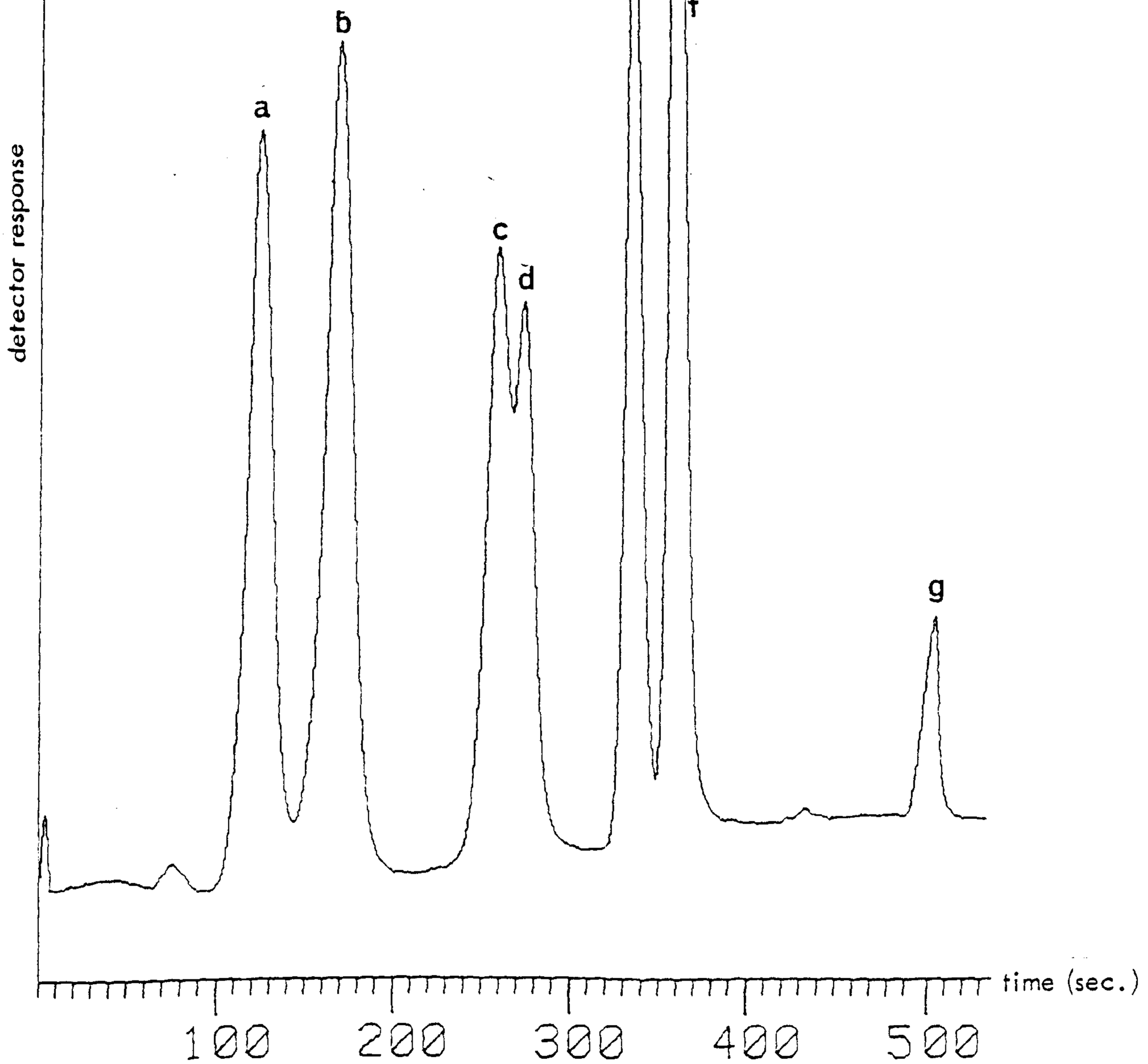


Fig 2.3.7. Computer printout showing standard compounds related to 4-acetamidobiphenyl separated by HPLC

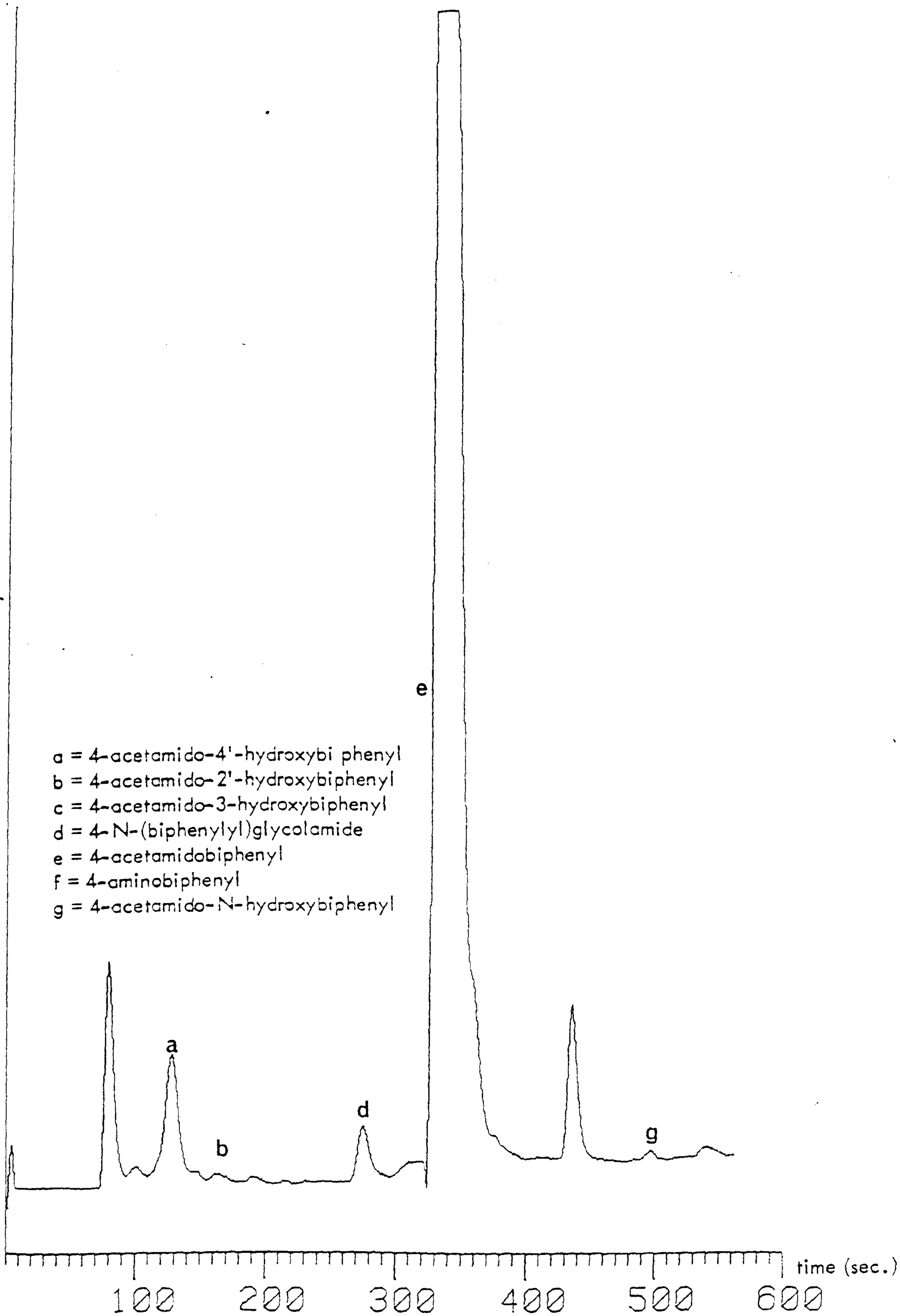


Fig. 2.3.8. Chromatogram of extract from a microsomal incubate of 4-acetamidobiphenyl using rabbit hepatic tissue

a = 4-acetamido-4'-hydroxybi phenyl
b = 4-acetamido-2'-hydroxybiphenyl
c = 4-acetamido-3-hydroxybiphenyl
d = 4-N-(biphenylyl)glycolamide
e = 4-acetamidobiphenyl
f = 4-aminobiphenyl
g = 4-acetamido-N-hydroxybiphenyl

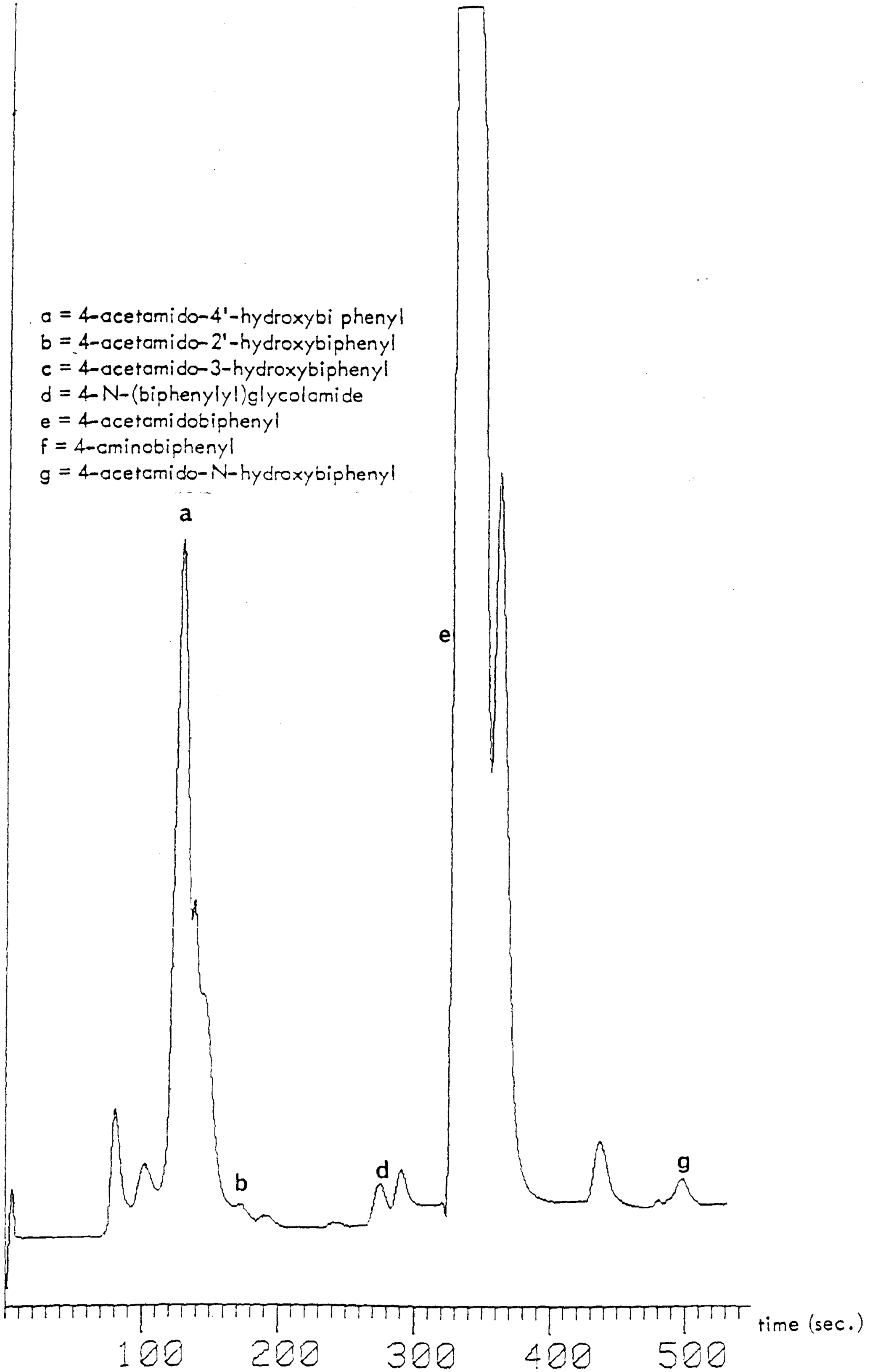


Fig. 2.3. 9. Chromatogram of extract from a microsomal incubate of 4-acetamidobiphenyl using hamster hepatic tissue

effect was caused by the inclusion of fluoride in the incubation media. It was decided to use twenty minutes' incubation time for all further work.

The influence of tissue and substrate concentration are recorded in Table 2.3.8. Increasing the tissue concentration caused an increase in metabolism at all sites of oxidation of both substrates; however, this increase was not linear up to microsomes derived from 0.75g or original liver, and it was therefore decided to use microsomes from 0.25g of liver for all further work. Increasing the apparent substrate concentration had little effect on any site of oxidation of either substrate (Table 2.3.8). This is perhaps not surprising as even at the lowest concentration the compounds are probably sufficient to saturate the solution and the oxidative enzymes. In all further work a substrate concentration of 5mM was used.

Using the conditions established above, a comparative study on the ability of various species to oxidise 4-aminobiphenyl and 4-acetamido-biphenyl was carried out. The results are recorded in Table 2.3.9. Considerable differences were observed, hamster tissue being the most active in its abili ty to oxidise virtually all the sites on both substrates. In contrast, the dog generally had the poorest ability to metabolise the substrates.

The guinea pig, which possesses average ability to metabolise 4-aminobiphenyl by metabolic attack on the N and 4'- position, has only one-third to a quarter of the average ability to hydroxylate in the 3-position. In this species and the dog no evidence could be found to indicate that N-hydroxylation of 4-ace tamidobiphenyl had occurred.

Table 2.3.7 The influence of incubation time on the metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl by rabbit liver microsomes

incubation time (min)	4-aminobiphenyl				4-acetamidobiphenyl			
	site of oxidation				site of oxidation			
	4'	2'	3	N	4'	2'	N	CH_3
5	3.2	1.1	1.5	15.1	5.7	1.4	5.2	2.8
10	3.8	1.7	4.6	33.1	9.8	1.4	13.0	4.3
15	4.5	2.2	4.6	49.6	14.0	1.4	15.6	5.7
20	5.8	2.8	6.1	62.0	17.2	1.4	20.8	7.1
'20F^-	5.1	2.8	5.8	64.7	-	-	-	-

Results are expressed as nanomoles product formed during incubation at 37°

'20F^- indicates that 100 μmoles of sodium fluoride was incorporated into this incubation.

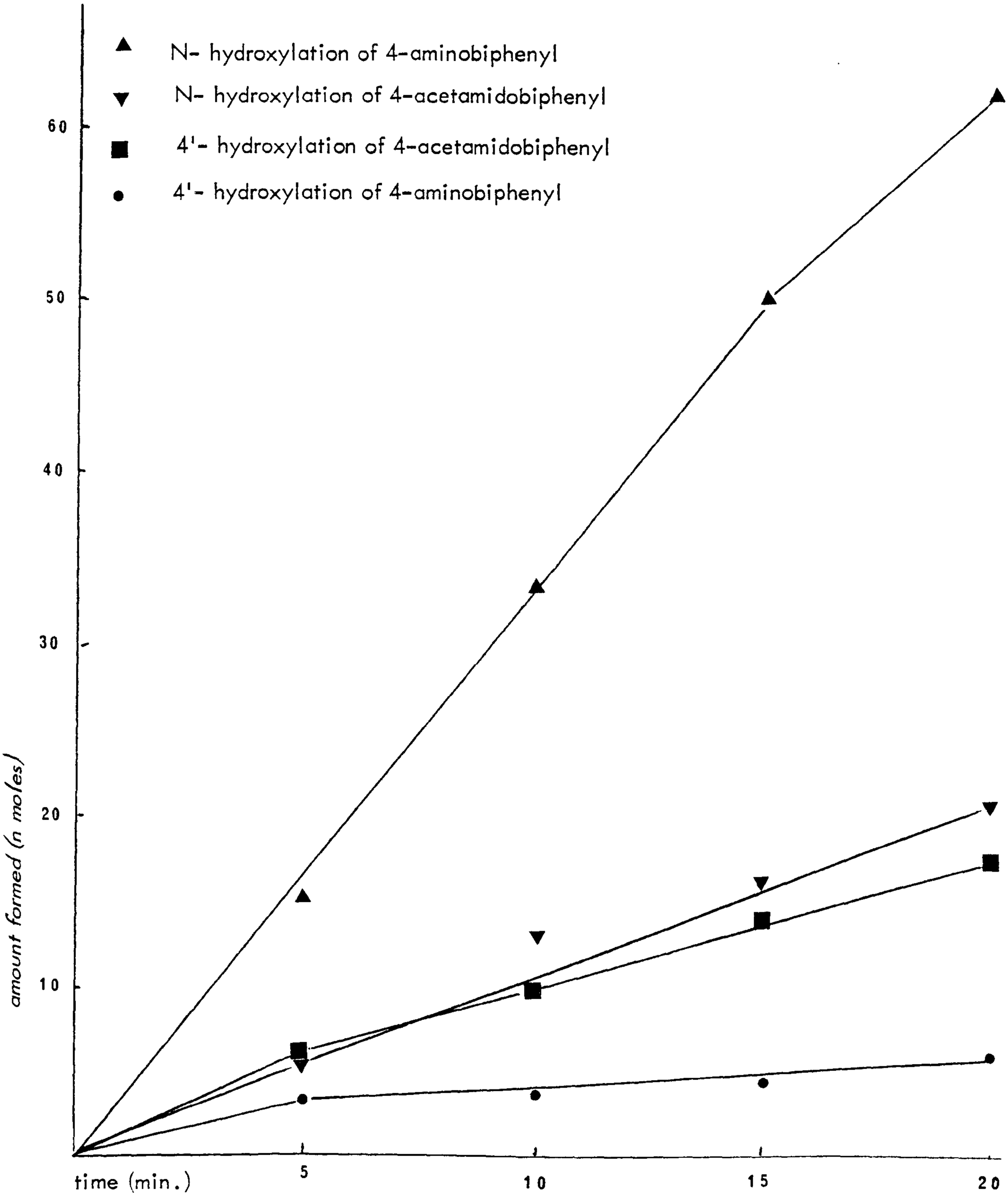


Fig. 2.3.10. The time course of N- and 4'-hydroxylation of 4-aminobiphenyl and 4-acetamidobiphenyl

Table 2.3.8 The influence on tissue and substrate concentration on the metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl by hamster hepatic microsomes

substrate	4-aminobiphenyl				4-acetamidobiphenyl			
tissue concn. (per incubate)	site of oxidation				site of oxidation			
	4'	2'	3	N	4'	2'	N	CH_3
0.125 g	5.5	2.6	24.3	63.9	67	4.2	10.4	3.6
0.25 g	6.4	5.3	43.8	113.2	122	7.0	23.4	5.0
0.50 g	9.2	9.6	72.9	182.6	195	9.8	46.8	8.5
0.75 g	10.1	11.4	82.6	173.4	251	15.4	70.2	8.5
substrate concn.								
5 mM	6.4	5.3	43.8	98.6	121	7.7	26.0	5.7
2.5 mM	5.5	5.3	38.9	104.1	116	6.3	28.6	6.4
1.25 mM	3.7	5.3	31.6	94.9	112	6.4	31.2	5.7

The results are expressed as nanomoles product formed during twenty minutes incubation at 37°.

Table 2.3.9 Inter-species differences in the metabolism of 4 -aminobiphenyl and 4-acetamidobiphenyl by hepatic microsomes

Substrate	4-aminobiphenyl[1]				4-acetamidobiphenyl[1]				
	site of oxidation				site of oxidation				
species	4'	2'	3	N	4'	2'	3	N	CH_3
rabbit	7.0	3.9	7.6	108.8	27.9	2.8	-	10.4	11.4
rat	3.1	1.7	9.1	64.7	33.6	2.1	-	-[2]	2.1
mouse	3.2	2.2	12.2	56.5	52.5	2.8	-	-[2]	5.0
hamster	8.3	6.1	66.8	316.8	260.8	5.6	-	41.6	9.9
guinea pig	9.0	1.1	3.0	70.2	61.5	2.8	-	-	8.5
dog	3.7	1.8	7.3	23.7	6.6	1.4	-	-	2.1

[1] The results are expressed as nanomoles product formed, during twenty minutes' incubation, by tissue derived from 250 mg of original liver.

[2] Examination of the chromatograms showed traces of material with the correct chromatographic properties, but this was too small to quantitate.

All species examined had the ability to metabolise 4-acetamidobiphenyl via an attack on the methyl constituent of the acetyl group, although considerable variation in activity was found; the rabbit and the mouse had about five times the ability of the rat and the dog.

The results obtained do not correlate very well with the carcinogenic activity of the substrates in a particular species. It is of interest that the hamster does have a very good ability to N-hydroxylate 4-aminobiphenyl and this compound is active in this species. However, the mouse and the dog are also sensitive to 4-aminobiphenyl yet they produce much less N-hydroxy compound.

The guinea pig, a species considered to be refractory to the effects of aromatic amines, is well able to N-oxidise 4-aminobiphenyl. It is clear that the further reaction of any compound formed (including reduction) will greatly influence the level of free compound actually available in any tissue, and it may be that further work is required to examine and quantitate these competing reactions.

From the point of view of inter-species difference it is interesting that the ratio of ortho to peri-para, i.e. 3- to 4'-, hydroxylation of an aromatic amine varies depending upon the species studied (see also Section 1.4), yet when the amine is acetylated ortho hydroxylation is completely abolished.

The availability of an assay system which allowed two substrates to be examined for multiple oxidation products allowed some further experiments on the enzymology of the oxidative processes involved.

Studies in which carbon monoxide was introduced into the incubation atmosphere were carried out and the results are shown in Table 2.3.10. In every case the presence of carbon monoxide inhibited the formation of products, suggesting the involvement of cytochrome P-450. However, the results were not uniform, and whilst increasing the ratio of carbon monoxide to oxygen caused a further decrease in the oxidation of 4'- and CH_3- oxidation of 4-acetamidobiphenyl and N-oxidation of 4-aminobiphenyl, this did not occur at the other sites of oxidation of 4-aminobiphenyl. In these cases an increase in the $CO:O_2$ ratio to 10:1 did, however, produce a further fall in the oxidation products. It is also interesting that in these experiments performed using a low oxygen tension - that is, only 4% oxygen in the atmosphere instead of the normal 20%, 2'- and N-hydroxylation of 4-acetamidobiphenyl could not be detected.

It was also of interest to pretreat animals with certain agents known to increase the activity of the microsomal enzymes. The results are presented in Table 2.3.11. Contrary to the expected increase, 3-methylcholanthrene caused a loss of enzyme activity at all sites of oxidation. This compound has previously been shown to increase the N-hydroxylation of 4-chloroaniline when given to rats (Uehleke 1967). The effect of methylcholanthrene was virtually mimicked by pretreatment with Arachlor 1254, except that this agent did not produce decreased 4'-hydroxylase activity using 4-acetamidobiphenyl as substrate. Phenobarbitone pretreatment did cause an increase in 4'-hydroxylation of both substrates and in N- and 3- hydroxylation of 4-aminobiphenyl; it was without effect on the N-hydroxylation

Table 2.3.10 The influence of carbon monoxide on the metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl by rabbit hepatic microsomes

substrate	4-aminobiphenyl				4-acetamidobiphenyl			
atmospheric composition	site of oxidation[1]				site of oxidation[1]			
$N_2 : O_2 : CO$	4'	2'	3	N	4'	2'	N	CH_3
96 : 4	7.0	3.9	9.1	68.9	17	-[2]	-[2]	10
82 : 4 : 10	3.8	1.7	4.6	35.8	14	-	-	9
72 : 4 : 20	3.8	1.7	4.6	31.7	10	-	-	4
52 : 4 : 40	2.6	1.1	3.0	20.7	12	-	-	5

[1] The results are expressed as nanomoles product formed, during twenty minutes' incubation, by tissue derived from 250 mg of original liver.

[2] These products were not detected under the conditions of low oxygen tension.

of 4-acetamidobiphenyl. The results give further support to the idea that differences exist in the enzymology of the N-hydroxylation of aromatic amines and amides (Gorrod, 1978a and b). The results are in agreement with those of Smith and Gorrod (1978) who reported that phenobarbitone pretreatment of rabbits caused an increase in the N-oxidation of aniline, 4-toluidine and 4-chloroaniline, whereas 3-methylcholanthrene was without significant effect. Phenobarbitone pretreatment has also been reported to increase the N-oxidation of 2-naphthylamine when given to dogs (Uehleke and Brill, 1968) but it was without effect on the N-oxidation of 4-aminobiphenyl in rats (McMahon 1979, personal communication). It seems that the response to an inducing agent is species-specific and extrapolation of data from one species to another is unwarranted.

Certain experiments were carried out in which substances which were known to have an effect on microsomal oxidations were incorporated into the incubation mixtures. The results obtained in the presence of these compounds are recorded in Table 2.3.12. As was expected the well-known inhibitors SKF525A and DPEA (McMahon & Mills, 1961) both inhibited oxidation at all sites on both substrates studied. Both SKF525A and DPEA inhibited the N-oxidation of the amine and the amide to the same extent, although DPEA produced the greatest inhibition with each substrate. This result indicates that these N-oxidations are not carried out by the flavoprotein system described by Ziegler, Poulsen and McKee (1971), as this was activated by primary alkylamines. These authors found that the best

Table 2.3.11. The influence of pretreatment of rabbits on the metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl by hepatic microsomes

substrate / inducing agent[1]	4-aminobiphenyl				4-acetamidobiphenyl			
	site of oxidation[2]				site of oxidation[2]			
	4'	2'	3	N	4'	2'	N	CH_3
none	5.8	2.8	6.1	62	21	2	8	10
3-methylcholanthrene	3.8	2.2	4.6	29	10	1	1	7
phenobarbitone	7.0	1.1	9.1	145	32	3	8	5
Arachlor 1254	3.8	2.8	4.6	33	19	2	4	7

[1] Conditions and dosage for the pretreatment of rabbits with inducing agents are given in the text.

[2] The results are expressed as nanomoles product formed, during twenty minutes' incubation, by tissue derived from 250 mg of original liver.

activator for this enzyme was n-octylamine, a substance which was also found to be a strong inhibitor of both aromatic amine and amide N-oxidation (Table 2.3.12).

The flavoprotein N-oxidase was also reported to be inhibited by N-1-naphthylthiourea (Ziegler & Mitchell, 1972) and strongly inhibited by methimazole (Poulsen, Hyslop & Ziegler, 1974). The former compound did inhibit the N-hydroxylation of 4-aminobiphenyl; however, methimazole was without effect on this process. These latter results also suggest that the N-hydroxylation of 4-aminobiphenyl is not mediated via the flavoprotein amine oxidase. Recently it has been shown that bromazepam can activate a number of cytochrome P-450 dependent N-oxidations (Gorrod 1978a, Smith & Gorrod, 1978) and it was interesting to note that an activating influence was also found on both the 3- and N-oxidation of 4-aminobiphenyl and the conversion of 4-acetamidobiphenyl into the glycolamide.

DABCO, a substance which has been used as a trapping agent for singlet oxygen (Ouannes & Wilson, 1968) had virtually no effect on any site of oxidation of either substrate, whereas adrenaline - which has the ability to react with superoxide anion (Ohnishi & Lieber, 1978) - virtually abolished the N- and 3- hydroxylation of 4-aminobiphenyl and the N- and CH_3-oxidation of 4-acetamidobiphenyl. This could be interpreted as indicating the involvement of superoxide as the active form of oxygen produced by microsomes and utilised in the oxidation of xenobiotics. Perhaps the most surprising results were those where 1-naphthylthiourea and

Table 2.3.12. The influence of some potential inhibitors or activators on the metabolism of 4-aminobiphenyl and 4-acetamidobiphenyl by a hepatic microsomal preparation derived from hamsters

substrate	4-aminobiphenyl				4-acetamidobiphenyl			
inhibitor or activator 10^{-3} M	site of oxidation				site of oxidation			
	4'	2'	3	N	4'	2'	N	CH_3
SKF 525A[2]	70	82	70	72	67	75	75	78
metyrapone	IA[1]	IA	143	75	101	IA	50	136
DPEA	55	33	33	32	13	20	33	25
N-1-naphthylthiourea	IA	IA	126	40	83	IA	IA	450
methimazole	IA	100	100	100	95	100	25	62
n-octylamine	78	85	63	13	13	40	0	75
bromazepam	IA	IA	218	118	112	IA	83	150
adrenaline	IA	118	7	0	1	40	0	0
α-naphthoflavone	264	51	85	40	89	40	IA	IA
DABCO	93	92	98	96	87	87	100	100

[1] IA indicates that the substance added or a metabolite formed from it interferes with the assay for a particular metabolite.

[2] Full names of compounds are given in text (Section 2.2.2.4).

The results are expressed as percentage of the activity found in the absence of any addition and measured after twenty minutes of incubation.

α -naphthoflavone were shown considerably to activate certain pathways. If these results can be substantiated in other species they may produce a useful tool to differentiate between the various forms of cytochrome P-450. At present it appears that all the oxidative pathways studied are mediated by cytochrome P-450. However, the different responses of each pathway to potential inhibitors, activators, inducing agents and change in oxygen tension suggest that more than one cytochrome P-450 is involved in the metabolism of these substrates.

It is also interesting to note that N-acetylation abolishes hydroxylation of the 3-position; this could be explained by considering the orientation of the substrates within the microsomal matrix. 4-Aminobiphenyl gives a type II binding spectrum with cytochrome P-450, whereas 4-acetamido-biphenyl shows a very weak type I interaction (Gorrod & Disley, unpublished observations). If this indicates that 4-aminobiphenyl is forming a sixth ligand on the haem moiety, then this could be the orientating effect necessary to direct oxidation to the 3- position. In conjunction with Dr. M. Kibby, molecular orbital calculations were carried out on 4-aminobiphenyl and 4-acetamidobiphenyl in order to see if any correlation could be found between any electronic parameters of a site within the molecule and its susceptibility towards microsomal oxidation. The data generated by this study are recorded in the Addendum.

In the present study no correlation could be found between the physico-chemical characteristics of a particular atom and its susceptibility to

microsomal oxidation. The calculations were carried out assuming the molecules to be in the ground state. It may be that further calculations, in which the electronic characteristics of the substrates in the excited states are determined, may provide a correlation.

SECTION 3. THE "IN VITRO" HEPATIC DEACETYLATION OF AROMATIC ACETAMIDO COMPOUNDS

3.1 INTRODUCTION

The deacetylation of aromatic N-acetamido compounds has been observed in a variety of animal species in vivo, and in certain tissue preparations in vitro. The considerable variation in the ability of species to carry out this hydrolytic reaction, together with the variation of subcellular location of enzyme activity depending upon the substrate, has presently made it impossible to make any generalisations regarding the enzymes involved or their substrate specificity.

The metabolism of aromatic acetamido-compounds may be different from that of the corresponding amine (see previous section and section 1.4). For example, aniline is hydroxylated at both the ortho and para positions of the nucleus, depending upon the species studied, whereas acetanilide is predominantly hydroxylated in the para position irrespective of the species studied (Parke, 1968).

Similarly, 2-naphthylamine appears in animal urine hydroxylated in the 1- or 6-position depending upon the species, whereas with 2-acetamidonaphthalene the 6 position is the major site of nuclear hydroxylation. This change in the site of hydroxylation is particularly pronounced in the dog, a species which allegedly lacks the ability to acetylate aromatic amines. In this species 2-naphthylamine is excreted hydroxylated in the 1 position to an extent in excess of 90% of the dose administered. When 2-acetamidonaphthalene is given to dogs less than 5% hydroxylation occurs in the ortho position, it occurs predominantly in the 6-(peri-para) position (section 1.4).

When aromatic amines are administered to experimental animals, N-acetylation occurs in most species; conversely when acetamido compounds are given or formed, some hydrolysis occurs. For each substrate an equilibrium amine/amide ratio is observed depending upon the species.

As the carcinogenic response from aromatic amines appears to be elicited without the requirement for prior acetylation (e.g. dogs are susceptible to aromatic amines, but cannot acetylate them), it seems that the rates of acetylation, and subsequent deacetylation, may be important not only in determining the site of metabolic hydroxylation, but also in indicating whether a compound is likely to be carcinogenic in a given species. It was for these reasons that studies of deacetylation and acetylation of some aromatic amines (reported in Section 4) were carried out.

Elson, Goulden and Warren (1946) found that acetanilide and phenacetin were almost completely deacetylated when given to rats, whereas Smith and Williams (1948) failed to detect deacetylation when these compounds were administered to rabbits. Similarly, N^4-acetylsulphamethazine was deacetylated when given to pigeons, dogs and cats, but apparently not deacetylated when administered to either rabbit or man (Krebs, Sykes & Bartley, 1947), indicating a marked species difference.

The in vitro hydrolysis of acetanilide was first reported by Michel, Bernheim and Bernheim (1937) who showed that this reaction was carried out by extracts of several rat tissues, the major activity being present in the liver and kidney. Acetanilide deacetylating activity was also present in hepatic extracts prepared from dog, cat, rabbit and ox. These authors compared results obtained from the hydrolysis of acetanilide with those from the hydrolysis of chloracetyl-l-tyrosine. They concluded that as the pH optima of these two enzyme activities were the same, and as the ratio of the activities were the same in various tissues, a common hydrolysing system was being utilised. This idea was not supported by Bray, James, Raffan, Ryman and Thorpe (1949), and Bray, James and Thorpe (1949), who

studied the hydrolysis of N-acetylglycine and acetanilide in liver and kidney preparations from many species, and concluded that an enzyme was responsible for the hydrolysis of aliphatic acetamides different to that hydrolysing aromatic acetamides. Bray, James, Thorpe and Wasdell (1950) extended these observations to other aliphatic and aromatic acetamido compounds, and noted differences in the sensitivity of these substrate groups to hydrolysis with regard to acetone and heat treatment of the tissue preparation. These results supported the idea that more than one enzyme capable of deacetylating acetamido compounds was present in mammalian tissues.

The first indication that deacetylation occurred during the metabolism of the carcinogen 2-acetamidofluorene came when it was observed that, following the administration of ^{14}C-acetyl-2-acetamidofluorene to rats, ^{14}C-carbon dioxide was detected in the expired air (Morris, Weisburger & Weisburger 1950; Weisburger, Weisburger & Morris, 1951). This was confirmed by Weisburger (1955) who showed that hydrolysis of 2-acetamidofluorene occurred in the presence of various tissue homogenates derived from rats or mice. Weisburger (1955) also studied the hydrolysis of other isomers of acetamidofluorene and found that, whereas the 2- and 4-isomers were hydrolysed at about the same rate, the 1- and 3-isomers were deacetylated at only about one fifth of the rate of the 2- and 4-isomers. In these experiments the incubation times used were very long (two and twenty two hours), so that the initial rates of hydrolysis could not be measured. The deacetylation of 2-acetamidofluorene by rat liver slices was confirmed by Peters and Gutmann (1955), who used radiochemical methods to follow the rates of hydrolysis. These authors also noted that rat liver slices were able to reacetylate the 2-aminofluorene formed, indicating that in the intact organ both

reactions were possible.

Argus and Ray (1959) examined the in vitro hydrolysis of a number of amide derivatives of 2-aminofluorene by 2N hydrochloric acid at 100°, and concluded that there was a relationship between the rate of hydrolysis of the amide linkage and the carcinogenicity of the compound, as those compounds which resisted hydrolysis were inactive in carcinogenicity tests.

Studies on the intracellular localisation of aromatic amide deacetylating enzymes have produced conflicting results. Early studies by Hollinger (1960a) showed that an acetanilide deacetylase was present in a microsomal preparation from rabbit liver. This enzyme was solubilised by treatment of the microsomal preparation by freezing and thawing. Chromatography on a calcium phosphate column and elution by phosphate buffer resulted in the separation of this enzyme from another protein, which had the ability to hydrolyse the amide bond in monoethylglycinxylidide but not that in acetanilide. Hollinger and Niklasson (1962) found that the acetanilide hydrolysing activity of the solubilised enzyme was enhanced in the presence of albumin.

In a series of publications on esterases Krisch and his colleagues described the hydrolytic properties of a number of proteins derived from various tissues. (Krisch, 1963a & b; Boguth, Krisch & Niemann, 1965; Benohr, Franz & Krisch, 1966; Benohr & Krisch,1967a). In these reports they showed that an enzyme with a very high esterase activity could be isolated from the microsomal fraction of kidney and liver of the pig. Whilst this enzyme was able to hydrolyse aromatic amides, the specific activity was very low compared to the specific activity obtained when aliphatic acetylamino acids or amino acid esters were used as substrates. A similar protein was isolated from beef liver, (Benohr & Krisch 1967b),

but differences were noted in the substrate specificity of this enzyme compared with that obtained from pig liver and kidney esterase. The beef liver enzyme had the additional catalytic property of transferring the amino acid residues from amino acid esters to form dipeptides. Benohr and Krisch (1967c) also showed that the enzyme from beef liver could be separated, using column chromatography, into two proteins, which were a monomer and dimer. These two forms of enzyme were interconvertible, the monomer having a molecular weight of 85,000, with one active centre, and the dimer 170,000, with two active centres. The enzymes were able to deacetylate a number of substituted anilides, but in no case did substitution significantly enhance the rate of hydrolysis compared to that found with acetanilide. In all the cases studied para-substitution reduced the rates of hydrolysis compared to that found with acetanilide. Ortho-substituents generally increased the rates of hydrolysis compared with those found with the para-isomers. The acetanilide deacetylase activity was potently inhibited by organophosphate insecticides, sodium fluoride and SKF 525A.

Bernhammer and Krisch (1965) investigated the hydrolysis of para-ethoxy-acetanilide (phenacetin) and para-carboxyacetanilide by the hog liver esterase. These authors found that whereas acetanilide was hydrolysed at about 391 n moles/min/mg, para-ethoxy acetanilide averaged about 125 n moles/min/mg, and para-carboxy acetanilide was hydrolysed at only 0.45 n moles/min/mg protein. The low activity observed with the latter substrate was attributed to its being more polar and less lipid soluble than the other substrates studied. It is of interest that a deacetylase enzyme located in the cell cytoplasm has been isolated from kidneys of rats, rabbits, mice and guinea pigs (Franklin, Bridges & Williams, 1971). This enzyme has the ability to hydrolyse para-carboxyacetanilide but not acetanilide.

In view of the variations both in substrate specificity and enzyme distribution in various species it was decided to study the deacetylation of acetamidc compounds derived from carcinogenic and non-carcinogenic amines. The deacetylation of acetamido compounds was followed by measurement of the parent amine by gas liquid chromatographic methods. The study was extended to various animal species in an attempt at correlation between rates of hydrolysis and carcinogenic activity of either the amine or its acetyl derivative in the species studied.

3.2 EXPERIMENTAL

3.2.1 Materials

1-Acetamidonaphthalene, 2-acetamidonaphthalene, 2-acetamidobiphenyl and 4-acetamidobiphenyl were all prepared by acetylation of the corresponding amine with acetic anhydride in pyridine. The acetamido compounds were recrystallised to constant melting point from aqueous ethanol. All acetamido-compounds had melting points consistent with those recorded in the literature; they gave one detectable substance when examined by gas liquid chromatography or thin layer chromatography. 2-Acetamidofluorene and 1-thioacetamido-naphthalene were purchased from Koch-Light Laboratories Ltd.

The corresponding aromatic amines were obtained and purified as described in Section 8.2. The arylhydrocarbons used were gifts from Dr.P.Sims. Reagent grade diethylether (B.D.H) was distilled before use. Sodium fluoride was obtained from B.D.H., SKF525A from Smith Kline and French Laboratories and E600 was a gift from Dr.D.Temple, Universitat Frankfurt.

3.2.2 Tissue preparations

Hepatic tissue fractions were obtained as described in Section 2.2 from adult male animals. In one case tissue from a 75 yr old human male, who had died four hours earlier from massive cerebral haemorrhage, was used.

3.2.3 Assay of aromatic amines

3.2.3.1 Gas chromatographic system

A Perkin-Elmer F11 gas chromatograph fitted with a two metre glass column o.d.$\frac{1}{4}$", packed with Silicone gum rubber SE30 2.5% on AW/DMCS treated Chromasorb G (80-100 mesh), was used throughout this work. The oven temperature was maintained at 190°, the injection temperature was 210°.

3.2.3.2 Optimum gas pressure.

The optimum gas pressures for the flame ionisation detector system were determined by injecting onto the column standard volumes of ethereal solutions of the amines using a glass micro-syringe fitted with a Cheney adaptor. A nitrogen pressure of 15 lbs/square inch was maintained during these measurements. After first determining the optimum hydrogen pressure, with the air pressure held constant, the air pressure was varied whilst the hydrogen pressure was held constant.

3.2.3.3 Determination of retention times for substrates and amines

Using the optimum conditions established in 3.2.3.2, ethereal solutions of the acetamido compounds and the corresponding aromatic amines were chromatographed and the retention times were recorded.

3.2.3.4 Choice of suitable internal marker

A number of aromatic hydrocarbons were examined as potential internal marker. They were subjected to chromatography as described in 3.2.3.3 and their retention times were recorded.

3.2.3.5 Linearity of detector response

Solutions of the aromatic amines in ether, containing the internal marker established in 3.2.3.4, were injected onto the gas chromatograph. Volumes ranging from 0.5 to 2.0 μ litre were injected using a micro-syringe fitted with a Cheney adaptor. The peak heights of the amines and the internal marker were measured.

3.2.4 Properties of arylacetamide deacetylase

3.2.4.1 Incubation procedure

All incubations were carried out in 50 ml conical flasks fitted with ground glass stoppers in a Gallenkamp shaking incubator at 37°.

3.2.4.2 Composition of incubate

The incubate consisted of tissue, usually derived from 0.5 g of liver suspended in 2 ml of tris/KCl, pH 7.4, and substrate, usually 10 μmoles in 3 ml phosphate buffer, pH 7.4.

3.2.4.3 Substrate specificity of deacetylase activity

Acetamido compounds and N-1-naphthylthioacetamide were incubated with rabbit whole liver homogenates at pH 7.4 as described in 3.2.4.1 and 3.2.4.2 for thirty minutes. The incubate was then extracted with ether, and the ethereal extract examined by chromatographic techniques for the presence of the deacetylated compound., as described in 3.2.3.

3.2.4.4 Subcellular distribution of deacetylase activity

Acetamido compounds were incubated with various mouse hepatic cell fractions at pH 7.4, prepared as described in Section 2.2. The aromatic amines produced during a thirty minute incubation were determined by gas-liquid chromatography.

3.2.4.5 Determination of pH optimum of deacetylase activity

Acetamido compounds were incubated with a rat hepatic microsomal preparation in the presence of phosphate buffers of differing composition in order to vary the final pH of the incubation system between pH 6.0 and pH 9.0. After thirty minutes' incubation the pH was adjusted to 7.4 and the aromatic amines produced were determined by gas-liquid chromatography.

3.2.4.6 Influence of microsomal protein concentration on the deacetylation of arylacetamides

Arylacetamides were incubated with a rabbit hepatic microsomal preparation. Flasks containing 10umoles ofsubstrate were incubated with microsomes equivalent to 0.125, 0.25, 0.5 and 1.0g of original liver tissue, for twenty minutes. The arylamines produced were determined by gas-liquid chromatography.

3.2.4.7 Influence of incubation time on the deacetylation of arylacetamides

Arylacetamides (10μmoles) were incubated with hepatic microsomal preparations derived from various animal species as described in 3.2.4.1 and 3.2.4.2. The enzymic reactions were stopped at either 10, 20, 30, 45 or 60 minutes of incubation, and the arylamine produced analysed by gas-liquid chromatography.

3.2.4.8 Influence of certain inhibitors on deacetylase activity

Arylacetamides (10 μmoles) were incubated with a rabbit liver microsomal preparation as described in 3.2.4.1 and 3.2.4.2. Control experiments were carried out in which no inhibitors were incorporated into the media. In other experiments various amounts of sodium fluoride, SKF 525A (diethylaminoethyl-diphenylpropylacetate hydrochloride) or E600 (diethyl-p-nitrophenylphosphate) were incorporated to investigate their possible inhibitory effects.

3.2.4.9 The influence of some metal ions on deacetylase activity

The influence of some metal ions on deacetylase activity was examined by their incorporation into the incubation media at a final concentration of 2μ moles/ml. The metals were all added as their chlorides. The substrate concentrations were 2μ moles/ml. In this experiment rat liver microsomes were used and the incubation time was thirty minutes.

3.2.5 Protein determination

Protein was determined by the Miller (1959) modification of the Lowry, Roseborough, Farr and Randall method as described in Section 7.2.3.

3.2.6 Partition coefficients

Partition coefficients of the arylacetamide substrates were determined by measuring their distribution between water and n-octanol. The solvents were saturated with the alternate phase prior to use, and equilibrium was established

by use of a bench rocking device for sixteen hours at room temperature. The initial and final concentrations of the acetamido compounds in the aqueous phase were determined using a Hilger ultraviolet spectrophotometer at the respective absorption maximum for each substance. Concentrations were determined by comparison with a calibration curve constructed from serial dilutions of standard stock solutions of the acetamido compounds.

3.3 RESULTS AND DISCUSSION

3.3.1 Development of a gas chromatographic assay for aromatic amines

The results obtained after varying the gas pressures to the chromatographic detector are summarised in Table 3.3.1. Increasing peak heights were observed upon decreasing the hydrogen pressure from 25 to 10 lbs/sq.inch. However, when hydrogen pressure below 10 lbs/sq.inch was used considerable instability of the detector response was observed. From these experiments it was decided to use a hydrogen pressure of 15 lbs/sq.inch for further work.

Under these conditions an increase in the pressure of air to the detector produced an increase in detector response. Instability of the instrument characteristics was observed at air pressures above 25 lbs/sq.inch, so that this pressure was used for subsequent work. The optimum hydrogen pressure was confirmed as 15 lbs/sq.inch in a further experiment when the air pressure was maintained at 25 lbs/sq. inch.

Using the optimised gas pressures, the retention times of various substrates and the corresponding aromatic amines were determined. Preliminary experiments indicated that an oven temperature of 190° was required in order to obtain sharp symmetrical peaks. The choice of this temperature allowed analysis of all the amines of interest as their retention times were short enough to be conveniently measured.

The retention times of the acetamido compounds of interest and the corresponding aromatic amines are recorded in Table 3.3.2.

Good separation of all the acetamido compounds from the corresponding aromatic amines was observed. Of the aromatic hydrocarbons examined for use as internal markers, only anthracene and phenanthrene had convenient retention times. Highly purified anthracene was commercially available and was chosen for further studies.

Table 3.3.1. The effect on the detector response of varying the gas pressures during chromatography of some aromatic amines.

Hydrogen Pressure*	Detector response in cm. 2ABP+	4ABP+	1AN+	2AN+	2AF+
25	17.3	8.5	15.1	11.9	4.6
20	34.1	10.7	22.1	24.0	7.4
15	48.0	16.0	35.0	32.5	13.0
10	52.0	22.5	35.5	36.5	9.3

keeping air at 15 lbs/sq.inch and nitrogen at 15lbs/sq.inch.

Air Pressure*	Detector response in cm. 2ABP+	4ABP+	1AN+	2AN+	2AF+
20	65.0	31.8	46.8	56.7	12.0
25	73.5	29.5	52.2	52.2	14.5
30	79.0	25.8	53.5	49.0	17.0

keeping hydrogen at 15 lbs/sq.inch and nitrogen at 15lbs/sq.inch.

Hydrogen Pressure*	Detector response in cm. 2ABP+	4ABP+	1AN+	2AN+	2AF+
26	10.2	3.9	10.3	7.4	2.4
22	12.3	7.6	11.5	12.0	3.6
18	17.8	9.4	16.8	13.8	5.0
14	18.8	10.3	17.8	17.0	5.9
10	16.5	6.6	15.5	14.2	3.8

keeping air at 25 lbs/sq.inch and nitrogen at 15 lbs/sq.inch

* All pressures are recorded as lbs/sq.inch.

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2ABP = 2-aminobiphenyl
4ABP = 4-aminobiphenyl
1AN = 1-aminonaphthalene
2AN = 2-aminonaphthalene
2AF = 2-aminofluorene.

Table 3.3.2. The retention times of some acetamido compounds, corresponding aromatic amines and some potential internal markers.

Compound	Retention Time * min. and sec.	
1-acetamidonaphthalene	5	15
1-aminonaphthylamine	2	03
2-acetamidonaphthalene	5	30
2-aminonaphthalene	2	10
2-acetamidobiphenyl	4	45
2-aminobiphenyl	2	20
4-acetamidobiphenyl	10	30
4-aminobiphenyl	3	32
2-acetamidofluorene	18	30
2-aminofluorene	6	58
1-thioacetamidonaphthalene	7	00
naphthalene	1	00
anthracene	4	10
phenanthrene	4	20
3-methylphenanthrene	6	45
pyrene	11	30

* Using 2.5% SE30 on AW/DMCS treated Chromosorb G (80-100 mesh); 2 metre x $\frac{1}{4}$" o.d. glass column at 190°.

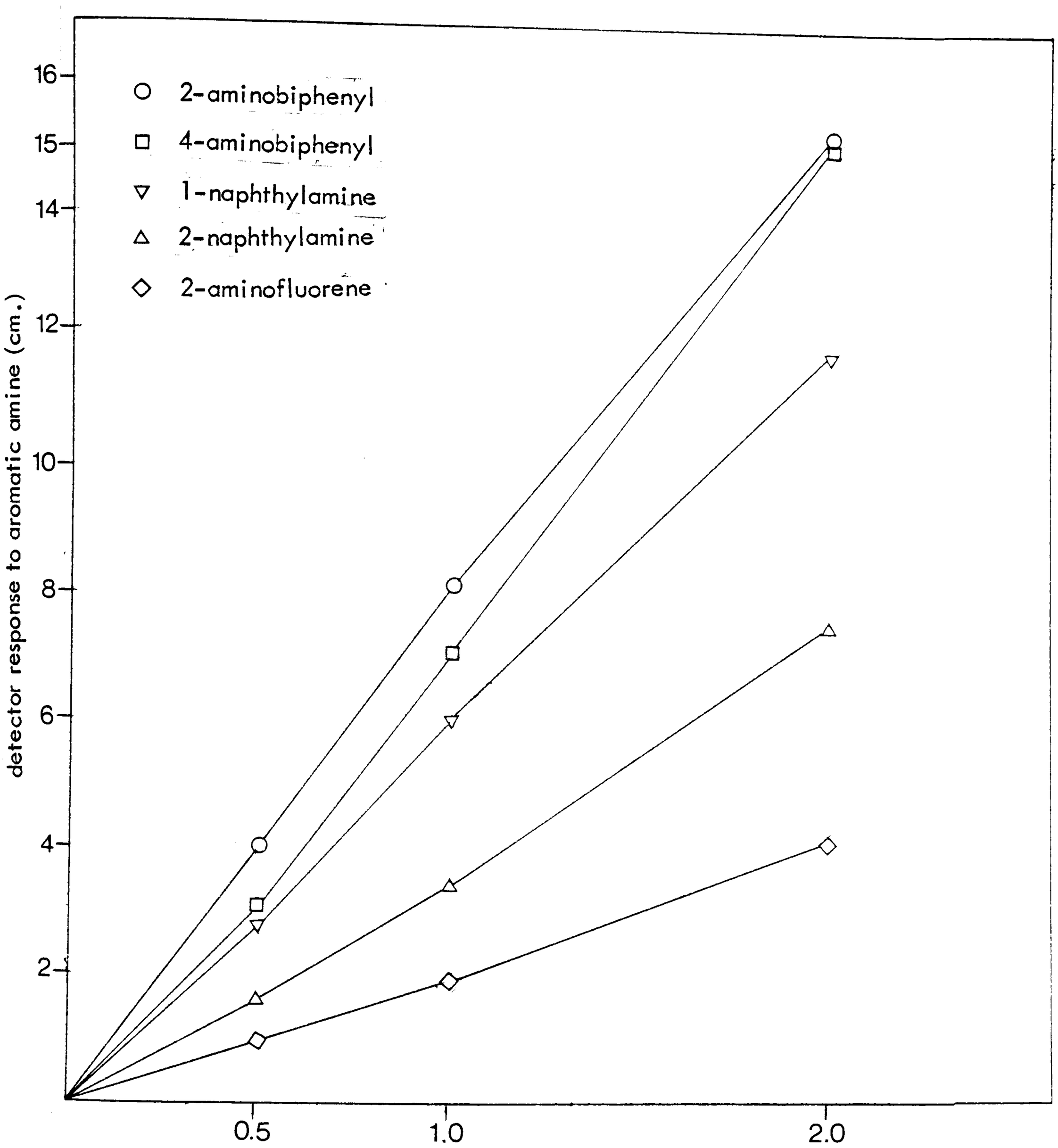


Figure 3.3.1. The relationship between detector response to aromatic amines and volume of solution injected.

The results of the experiments performed to check the linearity of detector response are shown in figures 3.3.1 and 3.3.2. When various volumes of standard solutions of aromatic amines are injected onto the chromatographic column a linear response was found up to 2.0 μ litre injected. Similarly, when the standard solutions contained both the amine and the internal marker a linear relationship was found, irrespective of the volume injected. It was concluded that the chromatographic system was suitable for the analysis of aromatic amines derived from acetamido compounds.

3.3.2 Final assay procedure used for the measurement of aromatic amines.

The deacetylation reaction was stopped by the addition of ether (10 ml) and cooling in an ice bath. A solution of anthracene (5 μmoles) in ether (1 ml) was added, and the aromatic amines extracted into the ether by multiple inversions during twenty minutes. An aliquot of the ethereal phase (1-2 μl) was injected onto the gas chromatograph and the peak heights of the amine and the anthracene measured. The oven temperature was maintained at 190° and the injection block at 210°. The column was 2 metre $\frac{1}{4}$" o.d. glass containing SE30 2.5% on AW/DMCS treated Chromosorb G (80-100) with nitrogen as the carrier gas at 15 lb/sq.in. The gas pressure to the F.I.D were hydrogen 15 lbs/sq.inch and air 25 lbs/sq.in. Using standard amounts of aromatic amines, a linear relationship between peak height ratio of amine to internal marker (anthracene) was obtained from 0.1 to 10 μ moles of amine.

The amount of amine produced by deacetylation was calculated by reference to standard curves constructed by the addition of known amounts of aromatic amines to microsomal suspensions and assayed as above.

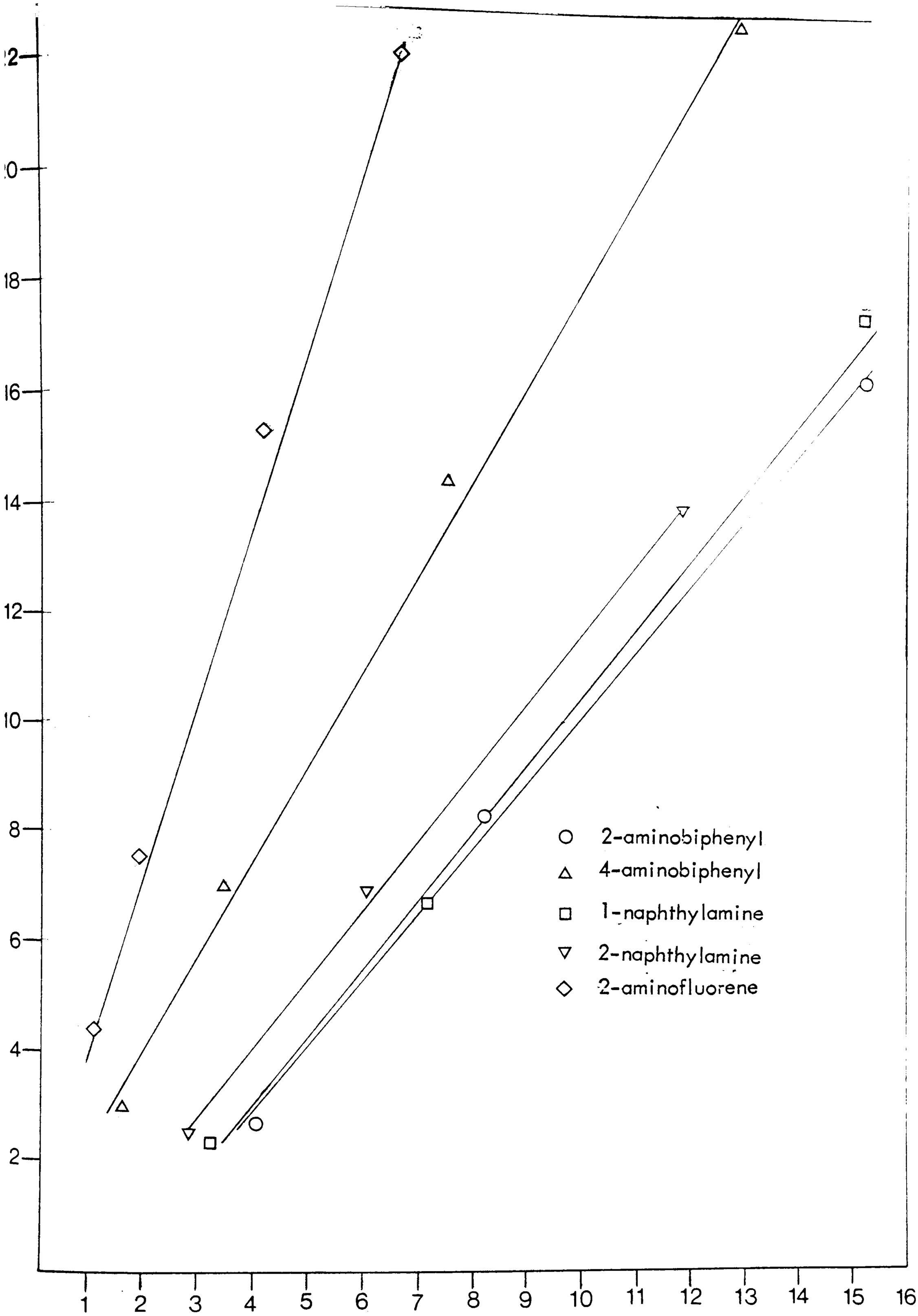


Figure 3.3.2. The relationship between detector response to aromatic amines and internal standard after injection of various volumes of solution.

3.3.3 Detection of aromatic amines derived from acetamido compounds

Following the incubation of acetamido compounds with tissue preparations the mixture was extracted with ether and the extract examined using the gas chromatographic system developed above. The ether extract was also examined using thin layer chromatographic plates coated with silica gel (0.25 mm) and developed with petroleum ether(b.p 80-100°) acetone mixture 4:2 by volume. In both gas chromatographic and thin layer chromatographic systems only the presence of the corresponding aromatic amine was detected in addition to the unchanged substrate. The aromatic amines had identical properties to authentic material in these chromatographic systems, and gave the same response to detecting reagents as known compounds on thin layer plates. No other compounds derived from the acetamido substrates were detected.

Using N-1-naphthylthioacetamide as a potential substrate, no evidence could be obtained for hydrolysis even though the techniques used were capable of detecting 0.1% of the substrate being hydrolysed.

3.3.4 Intracellular distribution of mouse hepatic N-arylacetamide deacetylase

The results obtained using four arylacetamides as substrates with various hepatic cell fractions are recorded in Table 3.3.3. Hydrolysis of all aryl-acetamides with all cell fractions was observed; however, the amounts of amines produced with the lysosome and mitochondrial fraction were small compared to that obtained using the microsomal fraction. Very little activity was found in the cell supernatant. The activity found in the microsomal fraction was not removed when the microsomes were washed by resuspension and recentrifugation. In each case the highest specific activity, calculated

Table 3.3.3. Intracellular distribution of mouse hepatic N-arylacetamide deacetylase

Substrate [1]	Fraction [2]	μ mol amine [3] liberated	Specific activity [4]
2-Acetamidobiphenyl	LM [5]	1.12	0.11
	M	3.75	0.98
	WM	3.55	1.54
	S	1.12	0.06
4-Acetamidobiphenyl	LM	2.12	0.29
	M	4.38	1.14
	WM	4.06	1.77
	S	0.14	0.007
1-Acetamidonaphthalene	LM	0.08	0.011
	M	0.56	0.15
	WM	0.50	0.22
	S	0.04	0.002
2-Acetamidonaphthalene	LM	0.22	0.03
	M	1.12	0.29
	WM	1.23	0.53
	S	0.045	0.002

1. 10 μmoles
2. LM = 7.3; M = 3.84; WM = 2.3; S = 18.7 mg protein
3. During 20 mins' incubation at 37°C with tissue equivalent to 0.5g of origional liver
4. μ moles/mg protein
5. LM = lysosome and mitochondria
   M = microsomes
   WM= washed microsomes
   S = supernatant

as μmoles/mg protein, was associated with the microsomal fraction and this was greatly increased using washed microsomes.

It can be assumed that washing the microsomes removes a certain amount of extraneous protein. The results clearly show that N-arylacetamide deacetylase activity is associated with the endoplasmic reticulum and it is probable that the activity found with the other cell fractions is caused by contamination of the fractions with endoplasmic reticulum. The above observations are in line with those of Krisch and coworkers cited earlier, who found acetanilide hydrolysing activity associated with the microsomal fraction in the livers of various species. The lack of activity found in the cell supernatant is in contrast to the observations of Franklin, Bridges and Williams (1971) using para-carboxy acetanilide as substrate, and show that at least two deacetylating systems must be present in the liver. It appears that the soluble enzyme preferentially hydrolyses polar acetanilides, whilst the microsomal enzyme is responsible for the hydrolysis of the non-polar compounds.

3.3.4 The pH optima of the microsomal deacetylating enzyme

The pH vs. activity curves for four enzyme substrates are shown in Fig.3.3.3. In all cases an increase in hydrolysing activity occurred between pH 6.0 and 8.5. At pH 9.0 a slight decrease in hydrolysing activity was found. Attempts to extend the pH range, in either the acid or alkali range, resulted in precipitation of the microsomal protein. The pH optimum for the enzyme activity was established as being in the region of 8.5, an observation in very good agreement with the data of Krisch (1963), who found that the microsomal acetanilide hydrolysing activity was maximal at pH 8.6 and Hollinger (1960b) who found a pH optimum of 8.5 for both a particulate and solubilised microsomal ethylglycinexylidide hydrolysing enzyme.

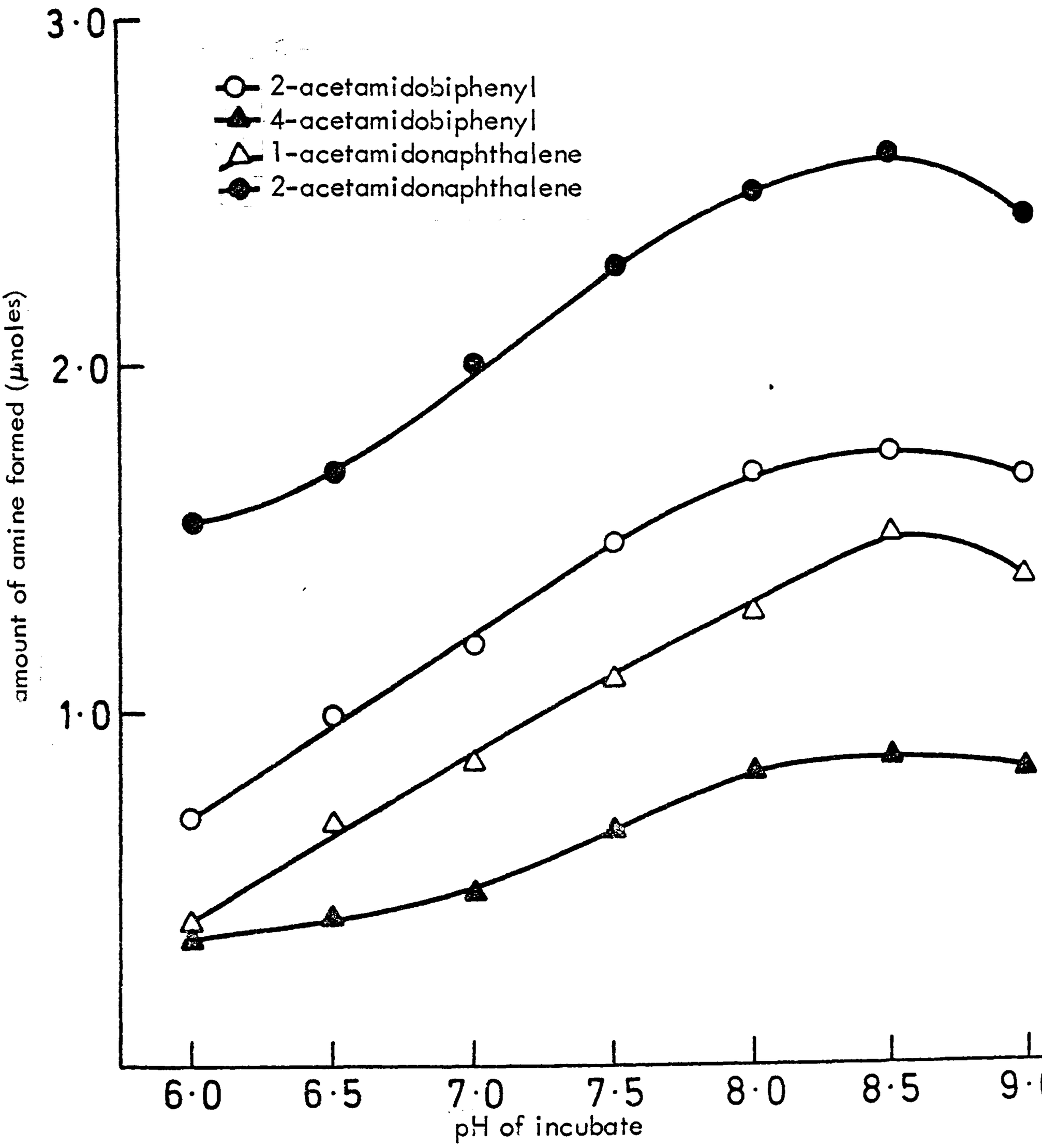


Fig.3.3.3. The effect of pH on the microsomal hydrolysis of N-arylacetamides.

The pH optimum for the soluble paracarboxyacetanilide hydrolysing enzyme has not been reported.

3.3.5 The influence of substrate concentration on the deacetylation of aromatic acetamides

The effect of increasing the substrate concentration on the hydrolysis of four acetamido compounds is shown in Fig.3.3.4. In the case of 2-acetamidobiphenyl and 2-acetamidonaphthalene increasing the concentration of the substrate, even up to 50μmoles per flask, was accompanied by an increased amount of amine produced. 1-Acetamidonaphthalene and 4-acetamidobiphenyl hydrolysis did not appreciably increase when the substrate concentration was increased beyond 10μmoles per flask.

Attempts to raise the substrate concentration of these latter two substances above 10μmoles per flask resulted in visible precipitation of the aryl acetamide from solution. In the light of this result it was decided to use 10 μmoles of each substrate per flask in further experiments.

3.3.6 The influence of incubation time on the course of hydrolysis of aromatic acetamido compounds by liver microsomes

The time course of hydrolysis of four acetamido compounds is shown for rabbit microsomes in Figure 3.3.5. It can be seen that whereas the hydrolysis of 4-acetamidobiphenyl and 2-acetamidonaphthalene gave a linear relationship with time up to sixty minutes of incubation, 2-acetamidobiphenyl and 1-acetamidonaphthalene both deviated from linearity early. This phenomenon was seen in all the species studied and created difficulties when comparing a series of substrates in various species. This was overcome by using short incubation times (see 3.3.7).

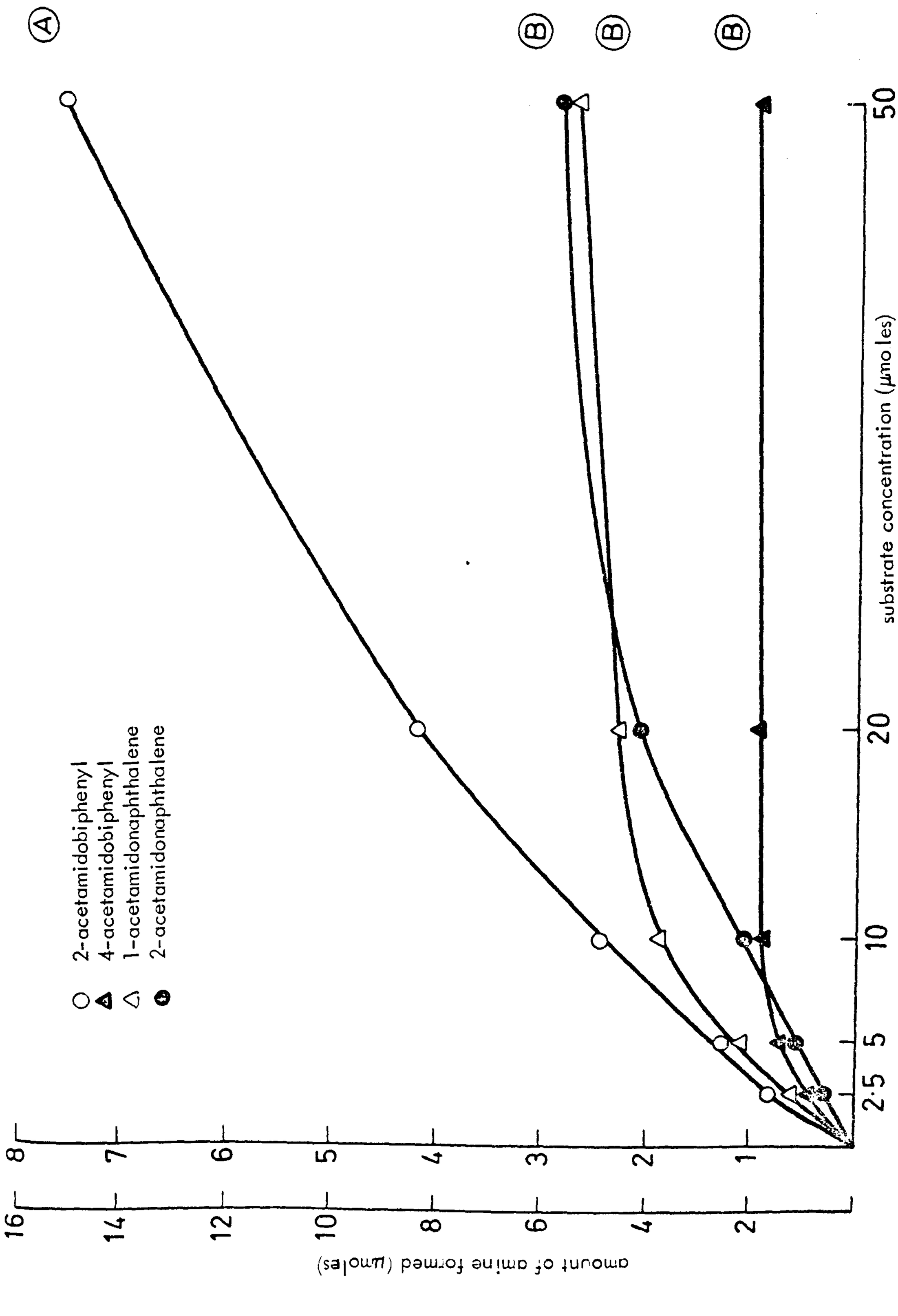


Fig.3.3.4. The influence of substrate concentration on the hydrolysis of N-arylacetamides

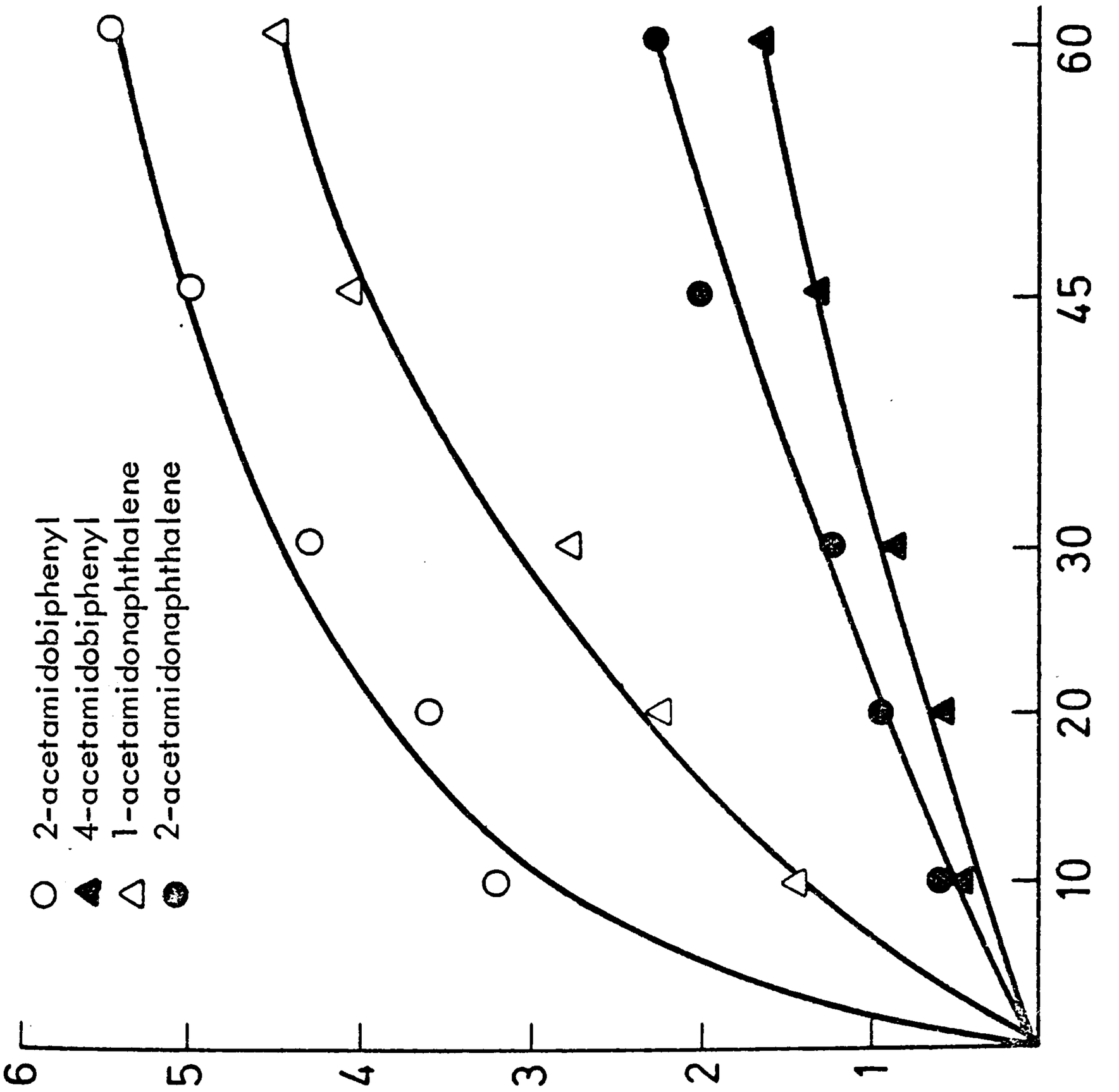


Fig.3.3.5. The time course of hydrolysis of N-arylacetamides by rabbit liver microsomes.

Control experiments in which tissue preparations were excluded or the microsomes were denatured by prior heating at 95° for twenty minutes demonstrated that no aromatic amine was produced by a non-enzymic reaction.

3.3.7 The influence of some metal ions and some potential inhibitors on microsomal deacetylation.

To clarify the situation with regard to the possible multiplicity of enzymic deacetylating systems, experiments were carried out in which the effects of metal ions on the deacetylation of 2-acetamidobiphenyl and 4-acetamidobiphenyl were observed. The results are shown in Table 3.3.4. No effect of sodium, manganese, calcium or iron on the hydrolysis of either 2- or 4-acetamidobiphenyl was detected, neither was their rate of hydrolysis stimulated by the incorporation of manganese ions. This is in agreement with Franklin, Bridges & Williams (1971) who found stimulation of the rat kidney deacetylase but not rat hepatic deacetylase by this metal ion. The inhibition of deacetylase activity by copper, zinc and mercury is in agreement with Nimmo-Smith (1960), and Franklin, Bridges & Williams (1971).

In contrast to the inhibition of hydrolysing activity found with SKF 525A by Hollinger (1960), no effect was observed when this compound was added to incubates containing either 2- or 4-acetamidobiphenyl (Table 3.3.5). Bernhammer and Krisch (1965) found only a slight inhibition of the deacetylation of phenacetin by SKF 525A although acetanilide hydrolysis was inhibited at 10^{-4}M (Benohr & Krisch, 1967b). Strong inhibition was observed in the presence of either sodium fluoride (Table 3.3.6) or E600 (Table 3.3.7.).

Table 3.3.4. The effect of metal ions on the deacetylation of 4-acetamidobiphenyl and 2-acetamidobiphenyl by rat liver microsomes.

Addition 2.0 μmoles/ml.	4-aminobiphenyl formed* (μmoles)	2-aminobiphenyl formed* (μmoles)
none	0.38	4.02
Mn^{++}	0.40	3.94
Na^{+}	0.39	4.05
Ca^{++}	0.39	3.95
Fe^{++}	0.40	3.85
Cu^{++}	0.30	2.98
Zn^{++}	0.25	1.52
Hg^{++}	0.02	0.05

*During thirty minutes incubation at 37°.

Table 3.3.5. The effect of SKF 525A on the hydrolysis of 2- and 4-acetamidobiphenyl by rabbit liver microsomes.

Addition		μ moles aromatic amine formed*	
		2-aminobiphenyl	4-aminobiphenyl
None		6.55	1.24
SKF525A	2×10^{-3}M	6.47	1.20
"	1×10^{-3}M	6.72	1.32
"	2×10^{-4}M	6.60	1.26
"	1×10^{-4}M	6.70	1.20

* During thirty minutes' incubation at 37°

Table 3.3.6. The effect of sodium fluoride on the hydrolysis of 2- and 4-acetamidobiphenyl by rabbit liver microsomes

Addition	2-aminobiphenyl formed*(μmoles)	% inhibition	4-aminobiphenyl formed * (μmoles)	% inhibitio
none	4.27	-	0.90	-
NaF 10^{-4}M	1.87	56.2	0.28	68.7
NaF 10^{-3}M	0.50	88.3	0.02	97.6
NaF 10^{-2}M	0.06	98.7	0.01	98.8

* During twenty minutes incubation at 37°

Table 3.3.7. The effect of diethyl-p-nitrophenylphosphate (E600) on the hydrolysis of 2- and 4-acetamidobiphenyl by rabbit liver microsomes.

Addition	μmoles amine formed *	
	2-aminobiphenyl	4-aminobiphenyl
none	6.27	1.12
E600 10^{-2}M	0	0
E600 10^{-3}M	0	0
E600 10^{-4}M	0	0
E600 10^{-5}M	0	0
E600 10^{-6}M	0.24	0.12

* During 20 minutes' incubation at 37°.

The inhibition observed in the presence of fluoride substantiates the work of others. Seal and Gutmann (1959) used fluoride as an inhibitor in their studies on the hydrolysis of 2-acetamidofluorene, as did Grantham, Weisburger and Weisburger (1965). Booth and Boyland (1964) had previously shown that the hydrolysis of 4-acetamidobiphenyl by rabbit hepatic microsomes was inhibited by fluoride. The complete inhibition of the microsomal deacetylating system observed in the presence of E600, even at low concentrations, is in agreement with the studies of Benohr and Krisch (1967b), who found no hydrolysis of acetanilide by a beef microsomal enzyme in the presence of 10^{-5} molar E600. This organophosphate also inhibits the deacetylation of N-hydroxy-2-acetamidofluorene (Irving, 1966).

3.3.8 Species differences in the deacetylation of aromatic acetamides

The results obtained for the comparative deacetylating ability of hepatic microsomes derived from common laboratory species and man are shown in Table 3.3.8. In the comparison of the isomeric acetamido biphenyls, in all cases except the mouse, the 2-isomer was deacetylated at a far greater rate than the 4-isomer. This is of particular interest as this would mean that animals exposed to 2-acetamidobiphenyl are likely to be exposed to high concentrations of 2-aminobiphenyl metabolically derived from it, with the concomittant risk of amine toxicity. Conversely, animals treated with 4-acetamidobiphenyl would be expected to be exposed only to lower concentrations of 4-aminobiphenyl with a lower risk of amine toxicity. In the mouse, a species in which liver tumours are produced following exposure to 4-aminobiphenyl, the high deacetylating activity observed towards 4-acetamidobiphenyl indicates that this compound might also be carcinogenic as a result of its conversion to 4-aminobiphenyl.

Table 3.3.8. Species differences in the deacetylation of arylacetamides by hepatic microsomes

Species	4-acetamido-biphenyl	2-acetamido-biphenyl	2-acetamido-naphthalene	1-acetamido-naphthalene
Rat	0.40	4.50	0.45	0.40
Rabbit	0.54	3.20	0.62	1.45
Guinea Pig	0.40	3.64	0.75	4.75
Mouse	2.90	2.45	0.40	0.25
Dog	0.35	1.92	0.05	0.12
Cat	0.21	3.68	0.63	0.44
Human	0.39	1.05	0.54	0.12

The results are expressed as μmoles substrate hydrolysed per gram of liver during ten minutes' incubation at 37° from 10μmoles of substrate. The results are derived from duplicate experiments from at least two animals. The cat and human data are from duplicate experiments on only a single sample of tissue.

Exposure of dogs to 4-acetamidobiphenyl is known to produce tumours of the urinary bladder (a process which does not require an acetamido group), so that it is tempting to suggest that the rate of deacetylation of this compound, in this species, is high enough to produce carcinogenic levels of 4-aminobiphenyl. However, if this were so then as the rates of deacetylation observed in the other species were comparable, bladder tumours might also have been expected upon exposure to 4-acetamidobiphenyl.

The fact that this is not the case suggests that the dog is unique in either possessing a further metabolic pathway to the active carcinogen, or that it lacks a detoxication pathway so that the 4-aminobiphenyl levels remain high. The results obtained from investigations of these possibilities are discussed in sections 2 and 4.

Comparison of the rates of enzymic hydrolysis of the isomeric acetamidonaphthalenes shows that generally the non-carcinogenic 1-isomer is hydrolysed faster than the carcinogenic 2-isomer. The very low deacetylase activity found for 2-acetamidonaphthalene in the dog is consistent with the finding that dogs excrete very little of a dose of this compound as metabolites possessing a free amino group. In the one human liver preparation studied the rate of deacetylation of 2-acetamidonaphthalene was as high as that of the experimental animals examined, indicating that man exposed to this compound may be at a similar risk to those exposed to 2-naphthylamine.

In other experiments, not recorded here, it was found that 2-acetamidofluorene was deacetylated at a rate slower than any of the other acetamido compounds tested, whereas acetanilide was hydrolysed at a faster rate.

It should be noted however that this comparison of species and substrates is not strictly valid, as differences in solubility precluded working at a common saturating substrate concentration. The concentration used was saturating for 4-acetamidobiphenyl and the figures shown in Table 3.3.8 are indicative of the true potential of the hepatic microsomes to hydrolyse this substrate.

In the case of 2-acetamidobiphenyl and 2-acetamidonaphthalene, this is not the situation and, as can be seen from Figure 3.3.4, exposure of the tissue preparations to higher concentrations of these substrates would have produced a higher rate of hydrolysis. As the actual concentration of the substrates in the hepatic endoplasmic reticulum in vivo is not known, it is difficult to extrapolate from the in vitro to the in vivo situation. In a study of the metabolism of 2-aminobiphenyl in rats, Gorrod and Carey (1970) failed to find any urinary metabolites in which the amino group was acetylated. The high in vitro deacetylase activity found in these experiments suggest that even if 2-aminobiphenyl had been acetylated, the product would have been subjected to extensive hydrolysis.

3.3.9 Partition coefficients of some arylacetamido compounds

The octanol-water partition coefficients were determined as described in 3.2.6 and are presented in Table 3.3.9. It can be seen that comparison of the isomeric pairs of either acetamidobiphenyls or acetamidonaphthyls reveals large differences in the partition coefficients. In both the cases studied, the amide having an ortho substituent had the smaller partition coefficient. Conversely, those with a blocked para position were more lipid soluble. This partition coefficient appears to be further increased when rigidity is

introduced into the molecule; e.g. the introduction of a methylene bridge into 4-acetamidobiphenyl to give 2-acetamidofluorene doubled the partition coefficient.

Table 3.3.9 The octanol-water partition coefficients of some arylacetamides

Compound	Partition coefficient
1- acetamidonaphthalene	55 ± 2
2- acetamidonaphthalene	414 ± 50
2- acetamidobiphenyl	125 ± 25
4- acetamidobiphenyl	1250 ± 100
2- acetamidofluorene	2660 ± 30

The results presented in this section clearly show that there is present in animal hepatic tissues an enzyme which can deacetylate aromatic acetamido compounds. This enzyme activity is shown to be associated with the microsomal fraction of liver cells and is able to hydrolyse 1- and 2-acetamidonaphthalene, 2- and 4-acetamidobiphenyl, 2-acetamidofluorene and acetanilide to the parent aromatic amines. This system was not able to hydrolyse the thioacetyl compound studied.

The studies carried out with the inhibitors indicate that this enzyme is probably the same as that solubilised and purified by Krisch and his colleagues. Bernhammer and Krisch (1965) consider that this enzyme is probably identical

to ali-esterase. If this is the case then it is of interest that in addition to the very high hydrolytic activity shown towards esters of carboxylic acids and amino acids the enzyme also possesses a low deacetylase activity. Whether the same active centre is involved in all these activities is not at present known; however, similarities in response to inhibitors suggest that only one centre is involved. Contrary evidence comes from the work of Bray, James, Raffan, Ryman & Thorpe (1949), and Bray, James, Thorpe and Wadsell (1950), where they showed differences in the stability towards heat of the enzymes which deacetylate acetanilide and N-acetylglycine. Further, differentiation in regard to pH optima (Nimmo-Smith, 1960), inhibition by SKF 525A (Hollinger, 1960) or manganese stimulation (Franklin, Bridges & Williams, 1971) indicate that several different enzymic deacetylating systems must be present. The substrate specificity, tissue location and subcellular distribution of these various enzyme activities requires further study.

The present work shows that even when a limited number of structurally similar substrates are examined a considerable difference in their rates of hydrolysis by a given species is found. The situation becomes even more complicated when the substrates are hydrolysed by hepatic microsomes derived from different species. At the present time certain generalisations have become apparent which are helpful in understanding the role of deacetylation in the total metabolism of a foreign compound.

When a pair of isomeric arylacetamides are hydrolysed by the microsomal system it seems that the arylacetamide having an ortho substituent is more rapidly hydrolysed than the corresponding para substituted isomer. This is exemplified by a comparison of the rates of hydrolysis of 2- and 4-acetamido-biphenyl.

In this case it can be seen that the first compound of the pair is usually hydrolysed at a greater rate than the second compound. This concept gains support from a consideration of earlier in vitro studies of substituted aryl-acetamides. Bray, James, Thorpe and Wasdell (1950) examined the influence of various nuclear substituents on the rates of deacetylation by a rat liver preparation. In every case the presence of an ortho substituent (these authors examined $-CO_2H$, $-CONH_2$, -Cl and $-NO_2$) enhanced the amount of de-acetylation compared to the unsubstituted acetanilide, whereas the corresponding para substituent decreased it compared to acetanilide.

Using the purified enzyme derived from beef liver, Benohr and Krisch (1967b) showed that ortho-fluoracetanilide was hydrolysed slightly faster than acetanilide whereas para-fluoracetanilide was hydrolysed considerably slower than either acetanilide or the ortho-fluoro compound. These authors also showed that phenacetin (para-ethoxyacetanilide), para-nitroacetanilide and para-methoxyacetanilide were poor substrates for the enzyme compared to acetanilide. Results for the pig liver enzyme did not show such a marked effect as that observed for the rat and cow enzymes, and in these species the ortho-methoxy compound was a poor substrate compared to the para-isomer.

Bernhammer and Krisch (1965) compared the rate of hydrolysis of the para-carboxyacetanilide with the rates of para-ethoxyacetanilide and acetanilide, and showed that the acidic compound was an extremely poor substrate, being hydrolysed about one thousand times slower than the unsubstituted compound. These authors attributed this resistance to hydrolysis as being due to either the high water solubility of the substrate, or its possession of an ionisable group, thus preventing interaction with the enzyme. The results in this section indicate quite a good correlation between partition coefficient and

degree of hydrolysis by the microsomal enzyme. The compounds having the lowest partition coefficient (and hence the highest water solubility) were the compounds most rapidly hydrolysed. However, the complexities involved in studies of deacetylating systems are exemplified by the recent results of Jarvinen, Santtiand Hopsu-Havu (1971). These authors isolated two deacetylating enzymes from guinea pig liver microsomes, and showed that both enzymes deacetylated p-nitroacetanilide and p-carboxyacetanilide faster than acetanilide, and whilst one enzyme hydrolysed p-ethoxyacetanilide the other did not. One enzyme also hydrolysed 2-acetamidonaphthalene at twice the rate of the one isomer, whereas the other enzyme hydrolysed the 1-isomer five times faster than the 2-isomer.

These authors were also able to demonstrate that the two enzymes differed in their ability to deacetylate 2-acetamidofluorene and the corresponding N-hydroxylated derivative. One enzyme hydrolysed the N-hydroxy compound two hundred and sixty times faster than the amide, whereas the other hydrolysed the amide twice as fast as the hydroxamic acid. The high rate of deacetylation of N-hydroxy-2-acetamidofluorene compared to the amide had previously been reported by Irving (1966). In view of the complexities reported in this section it is difficult to assess the total role of deacetylation in the overall metabolism of aromatic amines. As the introduction of a hydroxyl group into the aromatic nucleus usually occurs ortho or para to the amine or acetamide, it would be expected that these substituents could affect the rates of hydrolysis. However, as hydroxylated compounds are often conjugated at a rate much higher than their formation (Booth & Boyland, 1957) and these conjugates are highly ionised, it may be that they are effectively removed from the environment of the deacetylating systems.

SECTION 4 THE "IN VITRO" HEPATIC ACETYLATION OF AROMATIC AMINES

4.1 INTRODUCTION

Whilst the acetylation of aromatic amines was recognised early as a metabolic pathway (Hensel, 1915; Ellinger & Hensel, 1914; Muenzen, Cerecedo & Sherwii, 1926), it was not until the introduction of the sulphonamides and subsequent metabolic studies that the importance of this reaction began to be appreciated. Marshall, Cutting and Emerson (1937), and Fuller (1937), showed that the aromatic amino group (N^4) of sulphanilamide was acetylated in vivo by a variety of animal species including man. Klein & Harris (1938), utilising rabbit tissue slices in vitro, showed that the liver was the major site of this metabolic reaction. Thereafter followed a period of investigation of the biochemical mechanisms involved in acetylation reaction. These studies culminated in the implication of coenzyme A (Lipmann, 1945; Kaplan & Lipmann, 1948) and ultimately in the isolation of acetyl coenzyme A (Fig.4.1.1a) as the acetyl donor in the majority of enzymic acetylation processes (Stadtman, Novelli & Lipmann 1951; Lynen & Reichert 1951; Lynen, Reichert & Rueff, 1951).

The aromatic amine acetylating system was at first shown to require both adenosine triphosphate and magnesium in addition to coenzyme A and acetate. Later it was realised that the function of the former components was in the biosynthesis of acetyl coenzyme A (Chou & Lipmann, 1952), and that acetyl coenzyme A:arylamine N-acetyl transferase required only the acetylated coenzyme for active transfer to an acceptor molecule (Tabor, Mehler & Stadtman, 1953).

Acetylcoenzyme A:arylamine N-acetyl transferase was extensively studied using pigeon liver fractions and sulphanilamide as the acceptor molecule. In vitro studies were later extended to other acceptors, notably para-nitroaniline and para-amino benzoic acid, by Tabor et al (1953), and to other species.

Weber and Cohen (1967) showed that in rabbit liver the acetylating enzyme was localised in the cell cytoplasm, and Govier (1965) found the activity to be unique to the cells of the reticuloendothelial system of rabbit liver, and not to be associated with the hepatic parenchymal cells. Govier (1965) also demonstrated the presence of acetylcoenzyme A: arylamine N-acetyl transferase in other rabbit organs rich in reticuloendothelial cells, particularly the lung and spleen.

Parallel with these in vitro studies many investigations were carried out in vivo using many different species. Whilst the initial impetus for these studies was undoubtedly the clinical use of sulphonamides, the introduction of isoniazid into human medicine for the therapy of tuberculosis, soon provided a further stimulus. In their first investigation of sulphanilimide metabolism, Marshall et al. (1937) showed that both man and rabbit were able to acetylate this compound, whereas the dog was not able to carry out this reaction. Subsequently, Marshall (1938) demonstrated that many species, including fish, chicken, mouse, rat, guinea pig, cat, pig, cow, horse and monkey were all able to acetylate sulphanilamide, leaving the dog as an example of a species unable to acetylate aromatic amines.

The acetylation and subsequent deacetylation of the N^4-amino group of sulphonamides was examined by Krebs, Sykes and Bartley (1947), who concluded that the dog was able to acetylate sulphonamides, but that due to a high deacetylase activity in this species, the equilibrium lies towards production of compounds with a free aromatic aminogroup. Therefore, metabolites excreted in dog urine had a higher proportion of the deacetylated amino group compared to other species. This view was not accepted in a later paper by Marshall (1954) who emphasised that in his opinion the dog lacks

the acetylcoenzyme A acetyl transferase capable of acetylating either sulphanilamide or sulphamethazine. A further paper on the lack of acetylcoenzyme A: arylamine N-acetyl transferase activity in the dog by Leibman and Anaclerio (1962) implicates the presence of an inhibitor of the enzyme in this species. These authors failed to find any arylamine N-acetyl transferase activity in slices, minces, homogenates or acetone powder extracts of dog liver. These preparations were able to acetylate the aliphatic amino-group glucosamine suggesting the presence of a variety of acetylcoenzyme A: acetyl transferases whose specificity depends upon the nature of the amino substrate. The concept of multiple enzymes able to carry out acetyl transfer to different acceptor groups has been studied by Hease and Weber (1973) and reviewed by Weber (1973).

At the present time the exact nature of the acetyl transferase inhibitor present in dog liver described by Leibman and Anaclerio (1962) is unknown. The inhibitor is thermolabile and is inactivated by either trichloracetic or perchloric acids, and is thought to be a protein. The inhibitor also inhibits the transacetylation reaction, first described by Bessman and Lipmann (1953) whereby an acetyl group is transferred from a donor acetamido-compound (4-acetamidoazobenzene-4'-sulphonic acid) to an acceptor amine. In view of the observation by Steinberg, Cohen and Weber (1971), that the enzyme forms an acetylated intermediate (Fig.4.1.2) which then transfers the acetyl-group to an acceptor molecule, it is tempting to suggest that the inhibitor present in dog tissue is the acetyltransferase enzyme lacking a binding site for the aromatic amine acceptor molecule.

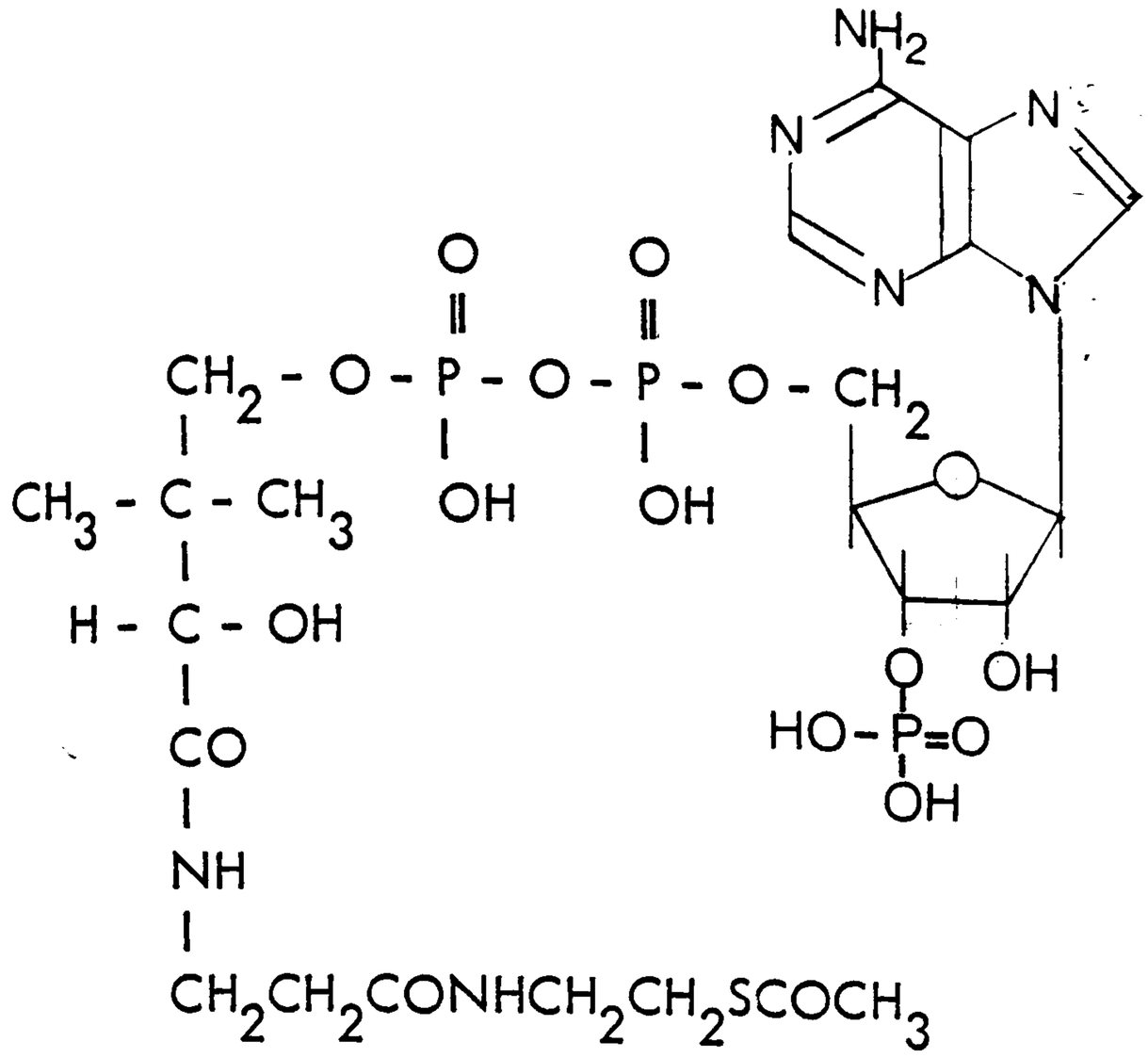
As 4-aminobiphenyl produces tumours of the urinary bladder when given to dogs (Section 1), and as acetylation of this compound alters the site of microsomal

hydroxylation of the aromatic system, it was thought desirable to confirm that the dog lacks the ability to acetylate 2- and 4-aminobiphenyl in vitro.

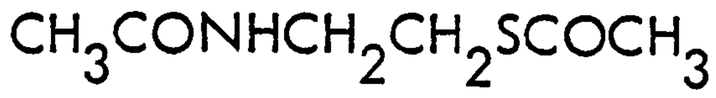
Genetic polymorphic distribution of acetyl transferase towards isoniazid, sulphamethazine and hydrallazine has been observed in man (Price-Evans & White, 1964), rabbits (Frymoyer & Jacox, 1963) and rhesus monkeys (Goedde, Schloot & Valesky, 1967). Because species and individual variation in susceptibility towards cancer induced by aromatic amines may be due to acetylation altering the site of oxidation, the acetylation of 2- and 4-aminobiphenyl in vitro by hepatic preparations from various species was examined.

One serious criticism that can be made against the bulk of the work reported on the in vitro acetylation of aromatic amines is the lack of specificity of the methods. Most authors rely on the disappearance of substrate, as measured by the Bratton and Marshall (1939) diazotisation and coupling colorimetric method, and assume that this quantitatively reflects acetamido formation. In other cases the experimental procedure contains both acetylating and deacetylating systems and attempts to study the former without either removal or inhibition of the latter can lead to erroneous results. For these reasons it is proposed to develop specific assays based on gas-liquid chromatography for the acetamido-compounds formed from both 2- and 4-aminobiphenyl.

Because of the structural similarity between N,S-diacetylcysteamine and the terminal moiety of acetyl coenzyme A, Goedde, Schloot and Valesky (1967) (Fig.4.1.1b) used the former compound as an acetyl donor in their in vitro study of the acetylating systems of the rhesus monkey. Because of the ease of preparation of N,S-diacetylcysteamine and the high cost of acetyl coenzyme A some experiments were carried out in which N,S-diacetylcysteamine was used as a cofactor in studies on the acetylation of aromatic amines.

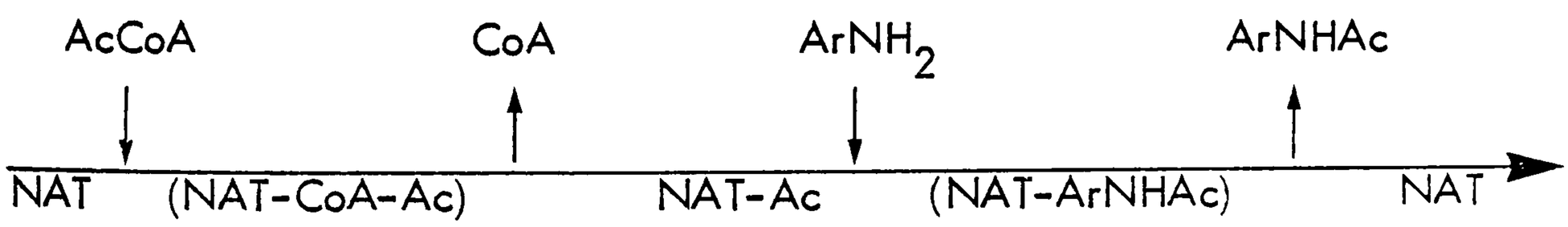


a) acetyl-coenzyme A.



b) N,S-diacetylcysteamine

Fig. 4.1.1. The structures of acetyl-coenzyme A and N,S-diacetylcysteamine



NAT = N-acetyltransferase

AcCoA = acetylcoenzyme A

$ArNH_2$ = aromatic amine

ArNHAc = acetylated amine

Fig.4.1.2. The proposed mechanism for the acetylation of aromatic amines (after Weber 1973).

4.2 EXPERIMENTAL

4.2.1 Materials

4.2.1.1 Substrates and metabolites

1- and 2-Naphthylamine, 1- and 2-acetamidonaphthalene, 2- and 4-aminobiphenyl and 2- and 4- acetamidobiphenyl were prepared and purified as described in the preceding section.

4.2.1.2 Cofactors

Coenzyme A, acetyl coenzyme A, acetylphosphate and adenosine triphosphate were obtained from Boehringer Ltd. N,S-Diacetylcysteamine (2-acetamidoethyl-thiolacetate) was prepared as described by Baddiley and Thain (1951); the product was purified by fractional distillation under reduced pressure, the fraction boiling at 123-125° at 0.1 mm being collected. This material solidified as long needles on cooling (m.p. 26°; lit. 26-28°).

4.2.1.3 Animals Animals used were the same species and strain as described in 2.2.1.4.

4.2.2 Methods

4.2.2.1 Tissue preparations. Cell fractions were obtained as described in 2.2.2.1.

4.2.2.2 Incubation procedures. Incubations were carried out in 25 ml conical flasks at 37 ± 1° as described in 2.2.2.2. Incubates contained aromatic amines as substrate, usually 5 μ mole, an acetyl donating system and a tissue fraction as a source of acetyl transferase. Variation of components of the incubate was carried out in order to determine optimum conditions for biological acetylation of aromatic amines. Incubates also contained sodium fluoride (100 μmole) to inhibit decetylase (see Section 3). In some experiments, incubation time was

also varied.

Typical incubates contained:

	Volume (ml)
Acceptor aromatic amine (5 μmole) in Cellosolve	0.2
Donor acetyl molecule (1 to 5 μmole)in water	0.1
Sodium fluoride (100 μmol) in water	0.2
Tissue preparation in tris/KCl buffer, pH 7.4 0.1M	1.0
Phosphate buffer, pH 7.4. 0.1M	2.0
total volume	3.5 ml

4.2.2.3 Qualitative experiments. Certain experiments were performed in order to examine the feasibility of the biological acetylation of aromatic amines being carried out in vitro. These experiments were terminated by the addition of diethyl ether, which was then used to extract the substrate and the metabolites from the aqueous phase. The ether extracts were evaporated to about 100 μl at 45° and examined for the presence of metabolites by chromatographic techniques.

4.2.2.4 Thin layer chromatography. Thin layer chromatography of the ether extracts were obtained using chromatography plates spread with Silica Gel G and various solvents. The chromatographic properties of the aromatic amines used as potential substrates and their acetylated derivatives were also determined using these chromatographic systems. The chromatographic properties of certain amines and their acetylated derivatives are recorded in Table 4.3.1.

4.2.2.5 Gas liquid chromatography. The ether extracts from incubates were examined by gas liquid chromatography using a Perkin Elmer F11 or F33 chromatograph fitted with glass columns and an F.I.D. The column used for routine

work, and for the quantitative determination of the acetamido compounds formed, utilised OV17 (3%) on Chromosorb G1 (80-100 mesh). The column was two metres in length with a $\frac{1}{4}$" o.d. In order to obtain good symmetrical peaks the oven temperature was maintained at 240° or 250° depending upon the compound of specific interest. During chromatographic analysis nitrogen carrier gas pressure was kept at 25 p.s.i., whilst the air pressure was 20 p.s.i., and hydrogen pressure was 17 p.s.i. The injection, port temperature was maintained at 325°.

In order to check the properties of the acetamido compounds formed, the extracts of the incubates were also examined using a 2 metre column containing OV1 (3%) on Gas-chrom. Q (60-80 mesh), with the same gas pressures as above. The columns were conditioned at 300° for 72 h prior to use and were silanised with hexamethyldisilazane (2 x 10 µl), leaving thirty minutes between each injection of this compound.

4.2.2.6 Quantitative assay for acetamido compounds. Enzymic activity was stopped by the addition of diethyl ether (4 ml) and internal standard (2-acetamidonaphthalene (1µmole in 1 ml phosphate buffer pH 7.4 containing 10% methanol). The flasks and contents were vigorously shaken and the contents transferred to Soviril screw-capped glass tubes which were then placed on a rocking device for five minutes. The ether phase was collected and placed in an evaporating tube. It was usually necessary to centrifuge the mixtures prior to this step in order to break any emulsion which had formed. The ether extractions were repeated with a further 3 x 4 ml of ether and the total ether extract collected, bulked and evaporated at 45° until about 100 µl remained. An aliquot of this concentrate (usually 2 µl) was chromatographed using a gas chromatograph fitted with a 2 metre glass column packed with

OV17 (3%) on Chromosorb G at an oven temperature of 240° for the determination of 2-acetamidobiphenyl and 250° for the determination of 4-acetamidobiphenyl. The other conditions were as described in 4.2.2.5.

4.3 RESULTS AND DISCUSSION

4.3.1 Chromatographic methods

The R_F and R_T values for the aromatic amines and the corresponding acetamido derivatives are recorded in Table 4.3.1. Both TLC and GLC gave good separation of the substrate from the acetamido derivative, allowing easy detection of the acetamido compounds if they were formed. No interfering substances were observed when "blank" incubates were examined in which the reaction had been stopped at zero time or in which the aromatic amine had been omitted. It was observed that the inhibitor SKF 525A had very similar chromatographic properties to those of 4-acetamidobiphenyl when examined using GLC and therefore it was not used in this work. Some spontaneous formation of acetamido compounds was observed when N,S-diacetylcysteamine was used in some experiments. This will be discussed later.

For routine purposes TLC plates were examined using UV light as the detecting agent. Additional confirmation of identity was obtained by spraying the plates with acidic p-dimethylaminobenzaldehyde or p-dimethylaminocinnamaldehyde (see Section 2) and allowing them to stand at room temperature overnight. On respraying with either dilute hydrochloric acid or acidic ferric chloride the acetamido compounds then gave the same response as the parent amines to these reagents.

In order to carry out routine analysis of 2- and 4-acetamidobiphenyl the oven temperature was maintained at 240° for the assay of 2-acetamidobiphenyl

Table 4.3.1 Chromatographic properties of some aromatic amines and their corresponding acetamido compounds

Compound	R_F^1			R_T^2 (mins)	
	A	B	C	OV17(3%)	OV1 (3%)
1-naphthylamine	0.42	0.85	0.49	2.0	1.0
1-acetamidonaphthalene	0.08	0.31	0.14	5.5	2.25
2-naphthylamine	0.31	0.80	0.44	2.25	1.1
2-acetamidonaphthalene	0.16	0.38	0.22	7.0	3.25
2-aminobiphenyl	0.68	0.95	0.63	2.0	1.0
2-acetamidobiphenyl	0.18	0.70	0.33	4.75	2.25
4-aminobiphenyl	0.31	0.81	0.46	3.5	1.75
4-acetamidobiphenyl	0.08	0.38	0.19	14.0	5.5

1. Using silica gel G uv_{254} at room temperature and allowing the solvent to ascend between 11 and 13 cm past the origin.
2. Using the gas pressures described in 4.2.2.5.
   OV17 column used at 240° with an inlet temperature of 300°
   OV1 column used at 200° with an inlet temperature of 275°.

A = Petroleum Ether (40-60), Acetone 9:1 v/v; B = Benzene, Methanol 9:1v/v

C = Toluene/methanol 95:5 v/v.

Table 4.3.2 Retention times[1] of 2- and 4-aminobiphenyl, 2- and 4-acetamidobiphenyl and the internal standard for the quantitative assay of the acetamido compounds

Compound	R_T^2 (min)	Compound	R_T^3 (min)
2-aminobiphenyl	1.5	4-aminobiphenyl	2.5
2-acetamidonaphthalene	4.5	2-acetamidonaphthalene	5.3
2-acetamidobiphenyl	3.0	4-acetamidobiphenyl	9.3

1. Using OV17 (3%) as described in 4.2.2.5 and 4.2.2.6
2. 240° 3. 250°

or 250° for the assay of 4-acetamidobiphenyl. This allowed a fairly rapid elution of the amide from the column. The retention times are recorded in Table 4.3.2. 2-Acetamidonaphthalene was chosen as the internal standard as it had suitable similar physicochemical properties to the compounds being assayed, was stable in solution for several months and had a suitable retention time for the analysis of both isomers of acetamidobiphenyl.

The quantitative assay developed gave a straight line relationship, using the peak height ratio method, up to 3.0 μmole 2- or 4-acetamidobiphenyl formed per incubate. Using this method 0.01 μmole of acetamido compound could easily be assayed.

4.3.2. Specificity of acetyl donor

Using a whole homogenate preparation from rabbit liver as potential acetyl transferase source and 1- and 2-naphthylamine and 2- and 4-amino-biphenyl as potential acceptor amines a variety of systems were investigated as to their usefulness as acetyl donor. Incubates were prepared containing either acetylphosphate (10 μmole), coenzyme A (1 μmole), coenzyme A (1 μmole) plus acetyl phosphate (10 μmole), coenzyme A (1 μmole) plus ATP (5 μmole) or acetyl conenzyme A (1 μmole). After incubating at 37° for thirty minutes, any acetamido compounds present were extracted as described in 4.2.2.3 and the ether extracts examined by TLC and GLC. None of the potential acetylating systems investigated appeared to be able to acetylate either 1-naphthylamine or 2-aminobiphenyl.

Positive evidence for the conversion of 2-naphthylamine and 4-amino-biphenyl to the corresponding acetamido compounds was found when coenzyme A was supplemented with either acetyl phosphate or ATP; no evidence for acetyla-

tion was obtained using only coenzyme A. Coenzyme A plus ATP appeared to produce more 2-acetamidonaphthalene and 4-acetamidobiphenyl than when acetyl phosphate was used in conjunction with coenzyme A. ATP in the presence of the tissue was unable to support acetylation of the substrates used. Strong evidence, i.e. large peaks at the correct retention time on the gas chromatograph using either column and strong discrete spots at the correct R_f in three solvent systems, was obtained for biological acetylation using 2-naphthylamine or 4-aminobiphenyl as acceptor amines.

Under the conditions used 1-acetamidonaphthalene and 2-acetamidobiphenyl could not be detected as metabolites of the respective amines. In these experiments the incubates did not contain fluoride ions, as it was thought that fluoride may inhibit either the formation of an active form of acetate or the transfer of acetate from the donor to an acceptor. In the light of the results from the previous section it is clear that any 1-acetamidonaphthalene or 2-acetamidobiphenyl formed could have been rapidly hydrolysed by the microsomal deacetylase as these were very good substrates for this enzyme. It was therefore concluded that future work would need to be carried out using acetyl coenzyme A as the acetyl donor.

4.3.3 Subcellular location of acetyl coenzyme A: arylamine-N-acetyl transferase

Using various subcellular fractions prepared from rabbit liver as potential sources of the acetyl transferase, acetyl coenzyme A (1 μmole) as the acetyl donor and 4-aminobiphenyl as the acceptor molecule, activity was determined after incubation for twenty minutes. The results which are presented in Table 4.3.3 show that the total activity of the homogenate can be found in the supematant preparation of the cell. The supematant which is derived from

Table 4.3.3. Sub-cellular distribution of acetyl coenzyme A: arylamine N-acetyl transferase

Tissue Preparation	4-acetamidobiphenyl formed (μ moles)[1]
whole homogenate	0.21
lysosomes and mitochondria	0.06
microsomes	0.022
washed microsomes	0.010
soluble supernatant	0.25

1. during twenty minutes at 37°.

Table 4.3.4. The effect of acetyl coenzyme A concentration on the acetylation of 4-aminobiphenyl

acetyl CoA (μ moles)	4-acetamidobiphenyl formed (μ moles)[1]
0	0.04
1	1.15
2	1.96
5	2.57
10	3.07

1. during twenty minutes at 37°

the cell cytoplasm, was used in all later experiments.

4.3.4 The effect of variation in acetyl coenzyme A concentration

The effect of varying the concentration of acetyl coenzyme A was investigated using a soluble enzyme preparation from rabbit liver. The enzyme preparation was equivalent to 0.5 g of original liver, and 4-aminobiphenyl (5 μmole) was used as acceptor molecule.

The results which are shown in Fig.4.3.1 and tabulated in Table 4.3.4 indicate that high levels of acetyl coenzyme A are needed to saturate the acetylating system. It was decided that 5 μmole of this cofactor per flask would be used in later experiments on species differences in arylamine acetylation.

4.3.5 The effect of variation in enzyme concentration

Using 4-aminobiphenyl and 2-aminobiphenyl (5 μmole) as acceptor molecules and enzyme prepared from guinea pig liver the effect of variation in enzyme concentration was studied. The acetyl coenzyme A concentration was 2 μmole per flask and the incubation time was twenty minutes. The results are shown in Fig.4.3.2 and Table 4.3.5.

Under the conditions used, the acetylation of 4-aminobiphenyl showed a linear relationship with the concentration of enzyme present up to the equivalent of 250 mg of original tissue per incubation. Doubling the enzyme concentration failed to produce a further increase. In agreement with the results of the qualitative experiments (reported in 4.3.2) only a trace amount of 2-acetamidobiphenyl was detected. It was decided that when comparing the potential of various species to acetylate aromatic amines tissue equivalent to 250 mg of original liver would be used per incubation flask.

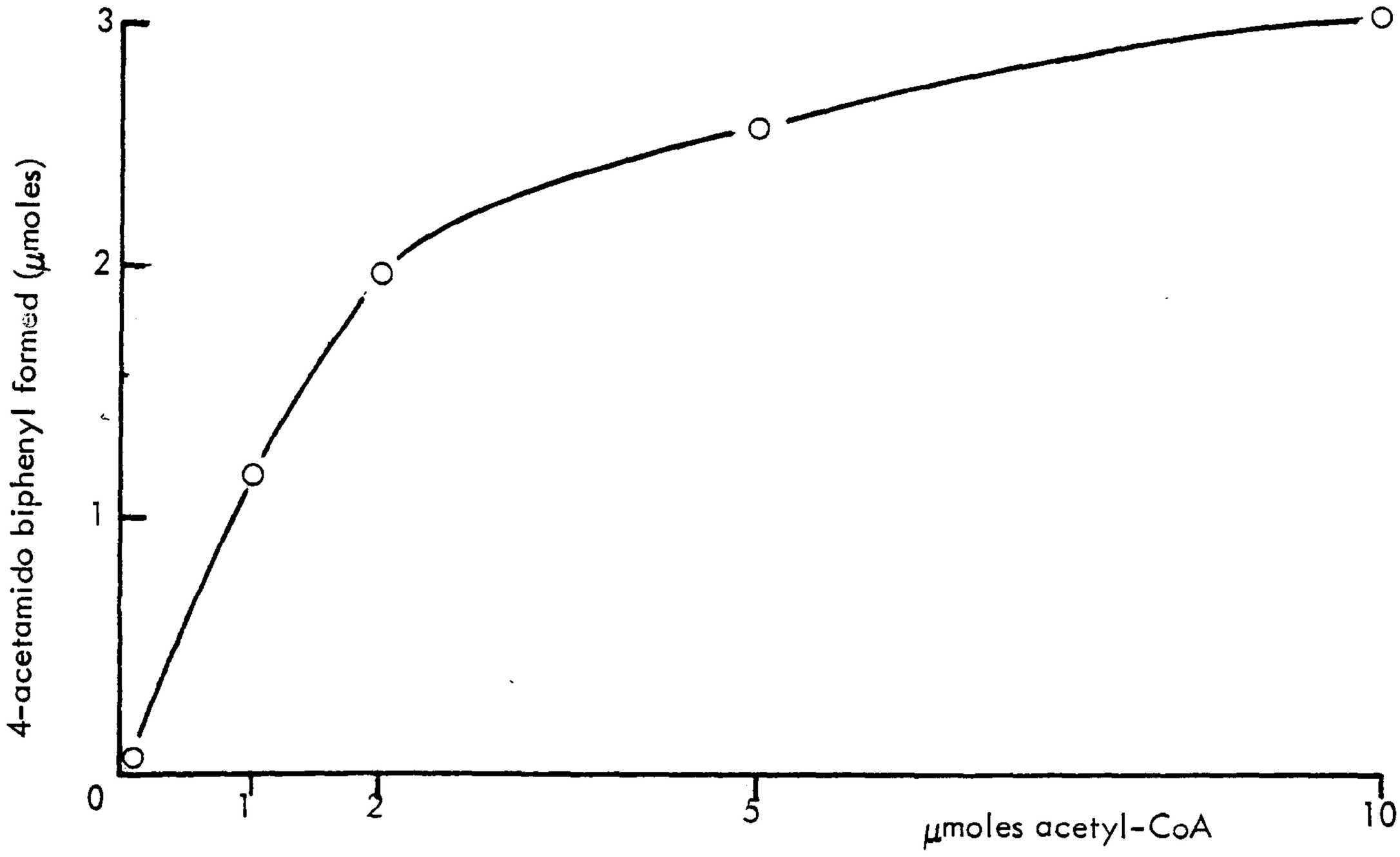


Fig. 4.3.1 The effect of acetyl coenzyme on the acetylation of 4-aminobiphenyl

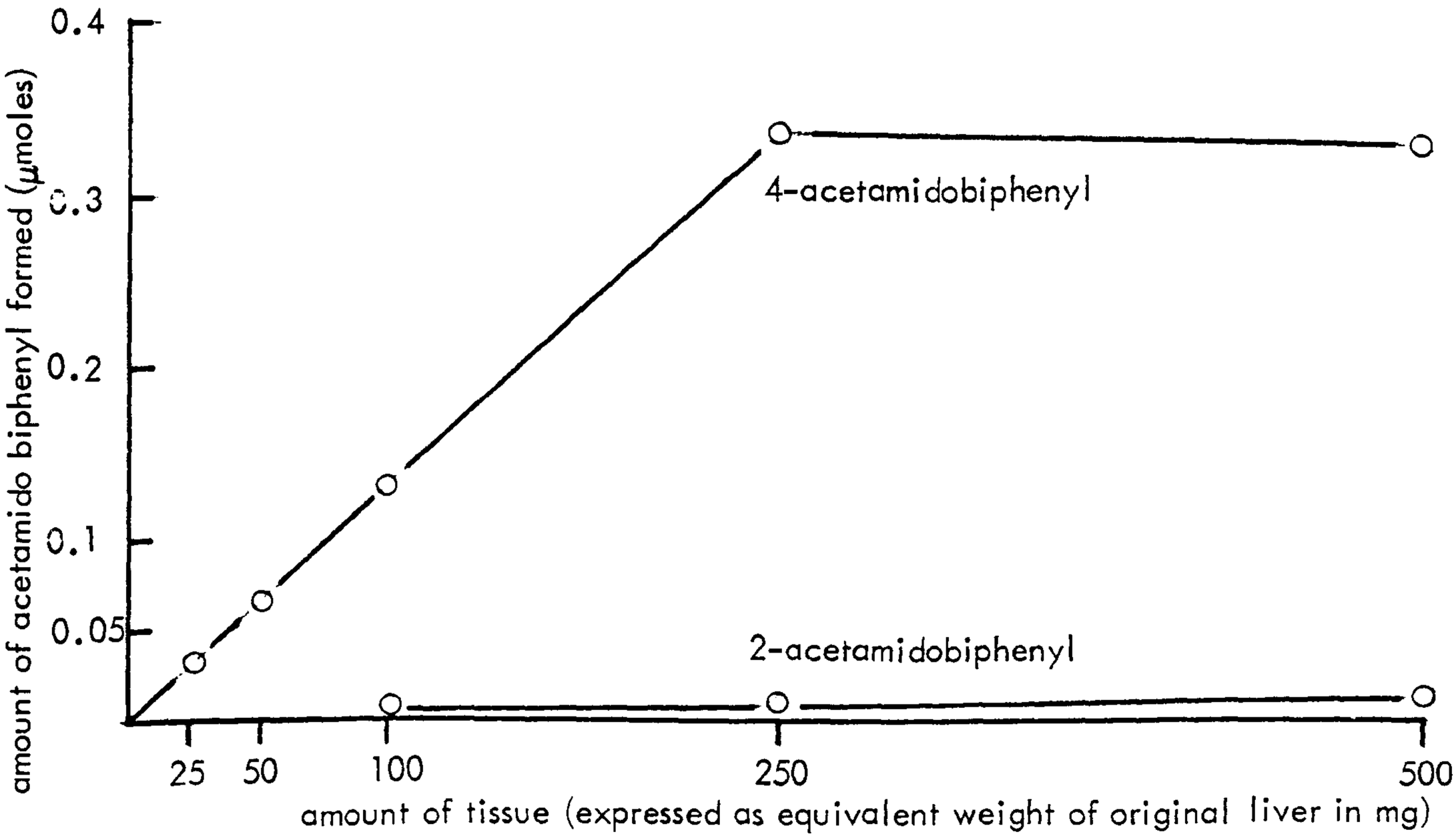


Fig. 4.3.2. The effect of tissue concentration on acetylation of 2- and 4-aminobiphenyl

Table 4.3.5 The effect of variation in enzyme concentration on the acetylation of 2- and 4-aminobiphenyl

Tissue ≡ mg of original liver	4-acetamidobiphenyl formed[1] (μmole)	2-acetamidobiphenyl formed[1] (μmole)
25	0.029	ND
50	0.058	ND
100	0.127	0.004
250	0.342	0.005
500	0.337	0.012

1. during twenty minutes at 37°

ND = none detected.

Table 4.3.6 Species variation in the acetylation of 4-aminobiphenyl

Species	4-acetamidobiphenyl formed (μmole)[1]
Rat	0.46
Mouse	0.73
Hamster	1.77
Guinea Pig	1.85
Rabbit	2.57

1. in the presence of supernatant fraction equivalent to 250 mg wet weight of liver during forty minutes at 37°.

4.3.6 Species differences in the acetylation of 4-aminobiphenyl

4-Aminobiphenyl (5 μmole) was incubated with tissue soluble fraction (equivalent to 250 mg of original liver) from various species in the presence of acetyl coenzyme A (5 μmole) for twenty minutes. The results, which are shown in Table 4.3.6, show that some differences in the ability of the liver from various species to acetylate 4-aminobiphenyl exist. The results are in general agreement with those of Lower and Bryan (1973) who examined the in vitro acetylation of 2-amino fluorene, and 2-aminonaphthalene as well as 4-aminobiphenyl. The results obtained in the present work and those of Lower and Bryan (1973) indicate that the guinea pig is one of the most active species in its ability to acetylate aromatic amines. The present results show that the rabbit and the hamster are also very good in this respect. Also in agreement with Lower and Bryan (1973) the lesser ability of the rat to acetylate 4-aminobiphenyl was confirmed. These authors were able to show that the dog was unable to acetylate 2-aminofluorene, 2-aminonaphthalene and 4-aminobiphenyl. Unfortunately dog liver was not available during the course of the experiments reported herein.

It is interesting that the two species which did not develop urinary tract tumours after exposure to 4-aminobiphenyl, i.e. rat and mouse, were the species with the lowest ability to acetylate 4-aminobiphenyl, whereas the species usually considered refactory to amine carcinogenesis was amongst the most active species in acetylating ability. However, it must be concluded that acetylation per se is unlikely to be related to amine carcinogenicity as the species with the highest demonstrable acetylating activity (the rabbit) and a species lacking in this activity (the dog) both contract bladder tumours after exposure to 4-aminobiphenyl.

When these results are examined in conjunction with those of Section 3 it can be seen that certain species are likely to excrete more of the free amine than the acetylated compound irrespective of whether 4-aminobiphenyl or 4-acetamidobiphenyl is administered. Thus it appears that the mouse has a high deacetylase activity plus a low acetylase activity. A similar situation obtains in the dog. The converse situation appears to occur in the rabbit and guinea pig which seem to have high levels of the 4-aminobiphenyl acetylating system with lower levels of the 4-acetamidobiphenyl deacetylating system. In these species 4-aminobiphenyl might be expected to be excreted predominantly as acetylated derivatives. As there is a difference in the sites of hydroxylation of 4-aminobiphenyl compared to 4-acetamidobiphenyl (Section 2) it is clear that these acetylating and deacetylating systems will play some role in determining the nature of the hydroxylated products excreted. It must be emphasised that this is only a very superficial study of the aromatic amine acetylating system.

It is known that the purified acetylating system exhibits rather complex enzyme kinetics (Hersch and Jenks, 1967; Jenne and Boyer, 1962; Steinberg, Cohen and Weber, 1971) and it may be that further studies will be needed to establish the complete picture of species differences.

It is also recognised that the ability to acetylate hydrazines and aromatic amines exhibits genetically controlled polymorphism, in humans (Price-Evans, Manley and McCusick, 1960). A similar situation appears to operate in rabbits (Frymoyer and Jaycox, 1963a) and rhesus monkeys (Goedde, Schloot and Valesky,1967), so that extension of these studies to a larger number of animals may have given rather different results. Incidentally, the same argument applies to carcinogenesis studies of aromatic amines in these species.

In the experiments reported in this section the acetylation occurred at only very low rates using 2-aminobiphenyl as the acceptor amine and either rabbit or guinea-pig tissue as acetyl transferase source. This observation, coupled with the observation of high deacetylase activity towards 2-acetamido-biphenyl exhibited by most species (section 3), would indicate that 2-aminobiphenyl is unlikely to be excreted as an acetylated metabolite. This is in agreement with the failure of Gorrod and Carey (1970) to find an acetylated metabolite of 2-aminobiphenyl when the amine was administered to rats. It has been suggested that the acetylation of aromatic amines is related to the electronic charge on the amino nitrogen (Perault and Pullman, 1963). It is true that both the electronic charge and the free valence of the 2-isomer is lower than that of the 4-isomer; however, both compounds have lower values than the values recorded for ortho-toluidine which is acetylated and higher than those recorded for ortho-nitroaniline which is not acetylated (Table 4.2.7)

Table 4.2.7. The electronic indices of some aromatic amines

Compound	Electronic charge on N	Free Valence of N atom	Reference
ortho-toluidine	1.852	1.027	Perault & Pullman (1963)
4-aminobiphenyl	1.842	1.014	Kibby)) see appendix
2-aminobiphenyl	1.838	1.008	Kibby)
ortho-nitroaniline	1.824	0.991	Perault & Pullman (1963

It would seem unlikely that such small differences involved could be the factor controlling whether acetylation can or cannot occur in biological systems. These results indicate that further work on this reaction is needed to establish the relationship between structure and susceptibility towards acetylation.

Because of the high cost of acetyl coenzyme A and the need to have a readily available pure acetyl donor in this and related work, N, S-diacetylcysteamine was prepared and examined as a potential acetyl source in the biological acetylation of aromatic amines. This compound was suggested by comparison of its structure with the terminal moiety of acetyl coenzyme A. (Fig.4.1.1) and had been previously used by Goedde, Schloot and Valesky (1967) in a study of the acetylation of both aliphatic and aromatic amines.

Following experiments in which 4-aminobiphenyl was used as an acceptor molecule and N,S-diacetylcysteamine was used as a potential acetyl donor, 4-acetamidobiphenyl was extracted and assayed according to the method described earlier (4.2.2.6). Irrespective of the time of incubation it appeared that a constant amount of 4-acetamidobiphenyl was formed. This phenomenon was further investigated by varying the amount of tissue present and keeping the time of incubation constant at twenty minutes. The assay again indicated that a constant amount of 4-acetamidobiphenyl was formed, even in the complete absence of tissue. These results suggested that N,S-diacetylcysteamine was able to act as a chemical acylating agent. Further work established that the acetylation of 4-aminobiphenyl by N,S-diacetyl-cysteamine occurred predominantly during gas liquid chromatography of the ether extracts, presumably due to the high temperatures needed for the chromatographic analysis of 4-acetamidobiphenyl. This "on-column" acetylation could be minimised by treating incubates with sodium hydroxide (2N, 4 ml) (McQuillin & Stewart, 1955) and allowing the mixture to stand at room temperature overnight before ether extraction and assay. Alkali

treatment did not appear to affect the concentration of 4-acetamidobiphenyl but did appear to destroy any surplus N,S-diacetylcysteamine. Because of these results it was decided that N,S-diacetylcysteamine could not be easily used as an acetyl donor in this work and its use was not continued with. These findings suggest that any study using N,S-diacetylcysteamine as an acetyl donor in which the resulting reaction mixture is exposed to changes of temperature may inadvertently be measuring non-enzymic acetylation as well as enzymic acetylation (if it occurs). The observation that N,S-diacetylcysteamine is able to react "on column" to produce acetamido compounds from 1- and 2-naphthylamine, 2-aminofluorene and 2-aminobiphenyl as well as 4-amino-biphenyl suggests that this compound may be developed into a reagent for producing derivatives for use in gas chromatographic analysis. Such a reagent may be useful where anhydrous conditions are difficult to achieve and would be free from the disadvantage of producing strongly acidic by-products which could damage alkaline treated column material.

SECTION 5 THE SYNTHESIS AND BIOSYNTHESIS OF 4-AMINO-3-BIPHENYLYL-β-D-GLUCOSIDURONIC ACID

5.1 INTRODUCTION

The metabolism of 4-aminobiphenyl studied by Bradshaw (1959) showed that hydroxylation occurs at the three position of the biphenyl nucleus. This hydroxylated metabolite was conjugated with either sulphuric or glucuronic acid prior to excretion in the urine. Further, the bladder implantation experiments of Bonser, Clayson and Jull (1956, 1958) and Allen, Boyland, Dukes, Horning and Watson (1957) implicated ortho-aminophenols, and hence this metabolite, as being the active carcinogen derived from 4-aminobiphenyl. As the corresponding sulphate conjugates were found to be inactive in bladder implantation tests, and resistant to enzymic hydrolysis, Boyland (1956) suggested that the glucuronic acid conjugates were the precursors giving rise to the carcinogenic ortho-aminophenols after hydrolysis by β-glucuronidase present in either urine or blood epithelial cells.

It was therefore of some interest to synthesise 4-amino-3-biphenylyl-β-D-glucosiduronic acid and to examine animal species variation in their ability to form this conjugate from its aglycone, as this may correlate with the susceptibility of the species towards bladder cancer produced by exposure to aromatic amines.

The reactions involved in the biosynthesis of various types of glucuronides have been reviewed by Dutton (1966). The enzyme responsible for the final step of the biosynthesis is located in the endoplasmic reticulum of cells, the highest activity being found in liver tissue preparations. This enzyme catalyses the transfer of glucuronic acid from uridinediphosphoglucuronic acid (UDPGA) to suitable acceptor molecules. The enzyme requires the presence of magnesium ions for optimal activity.

As animal species variations in the biosynthesis and the hydrolysis of 4-amino-3-biphenylylglucosiduronate may occur, some in vivo experiments to detect the presence of this compound were carried out.

The widespread use of an assay for glucuronyl transferase based on the conjugation of ortho-aminophenol (Dutton & Storey, 1962), suggested its use for the para-substituted ortho-aminophenol of interest in this study.

5.2 EXPERIMENTAL

5.2.1 Synthesis of Methyl(4-nitro-3-biphenylyl-tri -O-acetyl-β-D-glucosiduronate (I)

4-Nitro-3-hydroxybiphenyl potassium salt (3.2g) was refluxed in absolute ethanol (200 ml) for two hours with methyl(tri-O-acetyl-α-D-glucopyranosylbromide) uronate (5g), prepared as described by Bollenback, Long, Benjamin and Lindquist (1955). The reaction mixture was evaporated to dryness using a rotary film evaporator at 40°, and the solid material obtained extracted with ether,(3 x 500 ml). The ethereal extract was washed with 1N potassium hydroxide (total 900 ml), water, and then dried over anhydrous sodium sulphate. The material obtained on evaporating the ether solution to dryness was recrystallised from absolute ethanol to yield methyl (4-nitro-3-biphenylyl-tri-O-acetyl-β-D-glucosid)uronate; yield 1.2 g; m.pt. 139°, as pale yellow needles.

(Found: C, 57.1 ; H, 4.8; N, 2.8%; $C_{25}H_{25}O_{12}N$ requires C, 56.5; H,4.7; N, 2.6%). The product gave a strong positive test with the Tollens naphtho-resorcinol (see 5.2.8) reagent for glucuronides. After hydrolysis with either acid or alkali 4-nitro-3-hydroxybiphenyl was detected by thin layer chromatography.

5.2.2 Synthesis of Methyl(4-amino-3-biphenylyl-tri-O-acetyl-β-D-glucosid) uronate (II)

Methyl-(4-nitro-3-biphenylyl-tri-O-acetyl-β-D-glucosid)uronate (2.5g) in ethanol was treated with hydrogen in the presence of Adams platinum oxide catalyst until no further uptake of hydrogen could be detected. The reaction mixture was filtered and evaporated to dryness using a rotary film evaporator at 40°. The yellow product was recrystallised once from acetone and once from methanol to give nearly white needles of methyl-(4-amino-3-biphenylyl-tri-O-acetyl-β-D-glucosid)uronate, m.pt. 164°; yield 1 g. (Found, C,60.5; H, 5.3; N,3.2%. $C_{25}H_{27}O_{10}N$ requires C,59.9; H, 5.4; N,2.8%). This compound gave a positive Tollen s reaction (see 5.2.8) and 4-amino-3-hydroxybiphenyl on acid hydrolysis and gave coloured materials when acidified solutions were treated with sodium nitrite followed by alkaline β-naphthol.

5.2.3 Synthesis of 4-Amino-3-biphenylyl-β-D-glucosiduronic acid (III)

The product obtained after the reduction of 1.1 g of methyl(4-nitro-3-biphenylyl-β-D-glucosid)uronate as described above was dissolved in methanol (300 ml) and treated with sodium methoxide (5g) and left for 15 h at 0°. The reaction mixture was evaporated to about 50 ml at 40° in a rotary film evaporator and the residue applied as streaks across the origin of several sheets of Whatman 3MM chromatography paper. The papers were developed for 18 h, using butanol, propanol, 2N ammonia, 2 : 1 : 1 by volume, as solvent.

The area from R_f 0.24 to R_f 0.33 was cut out and the product was eluted with methanol. Concentration of this eluate at 40° in a rotary film evaporator afforded 4-amino-3-biphenylyl-β-D-glucosiduronic acid yield 75 mg as a pale buff-coloured powder. (Found, C 48.3; H, 5.1; N, 3.3%. $C_{18}H_{18}O_7 N Na. 2H_2O$ requires C, 51.6; H, 5.3; N, 3.3%).

The infrared spectrum of this material was compared with that of ortho-aminophenol β-D-glucosiduronic acid (Sigma Chemical Co. Ltd.,) in a Nujol mull using a Unicam S.P.200 Infrared Spectrophotometer.

5.2.4. The biosynthesis of 4-amino-3-biphenylylglucosiduronic acid.

A microsomal fraction prepared as described earlier (Section 2) from 17.5g of guinea pig liver was resuspended in isotonic KCl (17.5μl) and incubated for two hours at 37° with 50 mg UDPGA in 0.1M phosphate buffer (35 ml) containing magnesium chloride (0.02M), and with 20 μmoles of 4-amino-3-hydroxybiphenyl dissolved in 17.5 ml ascorbic acid (0.2%).

The incubations were carried out in ten 50 ml conical flasks each containing seven ml of the incubation mixture. The reaction was stopped and the protein precipitated by the addition of absolute ethanol (15 ml) to each flask. After centrifuging at 10,000xg for 10 mins the supernatant was concentrated to about 20 ml in a rotary film evaporator at 45°.

5.2.5 Chromatographic Methods

Paper chromatograms of the synthetic compound and material obtained in 5.2.4 were run descending on Whatman No.1 paper using the following solvent systems.

A. Butan-1-ol saturated with 2N Ammonia.

B. Butan-1-ol, Propan-1-ol, 2N Ammonia (2:1:1 by vol.)

C. Butan-1-ol, Acetic Acid, Water (12:3:5 by vol.)

The developed chromatograms were examined under ultra-violet light and with some detecting reagents used in Section 2. In addition, potassium dichromate (0.1M) in acetic acid (0.05M) followed by silver nitrate (0.1M) as described by Knight and Young (1958) was used to detect easily oxidised materials, and naphthoresorcinol (0.1%) in trichloracetic acid (10%) followed by heating the

sprayed chromatogram between two glass plates at 100° (Dutton, 1966) was used to detect glucuronic acid conjugates.

5.2.6 Comparison of colour formed after diazotisation and coupling of synthetic and biosynthetic materials.

The synthetic and biosynthetic 4-amino-3-biphenylylglucosiduronic acids were treated separately with nitrous acid, ammonium sulphamate and N-1-naphthylethylenediamine (as described in 5.3.4) and the visible absorption spectra obtained.

5.2.7 Enzymic hydrolysis of 4-amino-3-biphenylylglucosiduronic acid

The synthetic and biosynthetic 4-amino-3-biphenylylglucosiduronic acids were separately treated with either beef liver glucuronidase (Ketodase, Warner Chilcot Eastleigh, Hants), or bacterial β-glucuronidase (Sigma Chemical Co.) at 37° for 20 h at pH 4.8 in 5 ml stoppered tubes. Suitable controls without enzyme or including glucosaccharo-1:4- lactone (gift from D.Manson) at a concentration of 10^{-3}M were also carried through the procedure. After incubation, the tube contents were treated with 1 ml trichloracetic acid (7.5%) and after centrifugation to remove protein were treated with the reagents described in 5.3.4. Separate incubates prepared as above (5.2.7) were also examined by paper chromatography using the solvent systems and detection reagents described in 5.2.5.

5.2.8 "Tollen s" reaction of synthetic and biosynthetic material

Solutions of the synthetic and biosynthetic material (2 ml) were treated with concentrated hydrochloric acid (2 ml) and naphthoresorcinol (10 mg) in a boiling water-bath for 20 mins. The cooled reaction mixtures were extracted with ethyl acetate (1 ml).

5.2.9 Development of assay for 4-amino-3-biphenylglucosiduronic acid.

The coloured product obtained when 4-amino-3-biphenylylglucosiduronate was diazotised and coupled with N-1-naphthylethylenediamine (N.E.D.) was

examined to study the influence of time, temperature and pH by variation of one parameter. This was done to optimise conditions for an assay system for 4-amino-3-biphenylylglucosiduronic acid.

5.2.10 Development of enzyme assay.

The influence of 4-amino-3-hydroxybiphenyl and UDPGA concentrations, and the time of incubation, on the subsequent biosynthesis of 4-amino-3-biphenylylglucosiduronic acid were investigated in order to determine optimum conditions for the assay of UDPGA: 4-amino-3-hydroxybiphenyl glucuronyl transferase.

5.2.11 Comparison of UDPGA:4-Amino-3-hydroxybiphenyl glucuronyl transferase in various animal species.

Hepatic microsomal preparations from various species were assayed for UDPGA: 4-Amino-3-hydroxybiphenyl glucuronyl transferase using the methods developed in 5.2.9 and 5.2.10.

5.2.12 The excretion of 4-amino-3-biphenylylglucosiduronic acid by various animal species.

4-Amino-3-hydroxybiphenyl was administered (i.p.) as a solution in arachis oil to a rat, cat and guinea-pig. The rat and cat were given 25 mg in 0.5 ml oil, the guinea-pig 50 mg in 1 ml oil. The animals were mature males weighing 300 g (rat), 3000g (cat) and 500 g (guinea-pig). They were housed in metabolism cages for eighteen hours following administration of the amine and urine collected.

The urine was examined using the chromatographic solvents and detection reagents described earlier (5.2.5).

5.3 RESULTS AND DISCUSSION

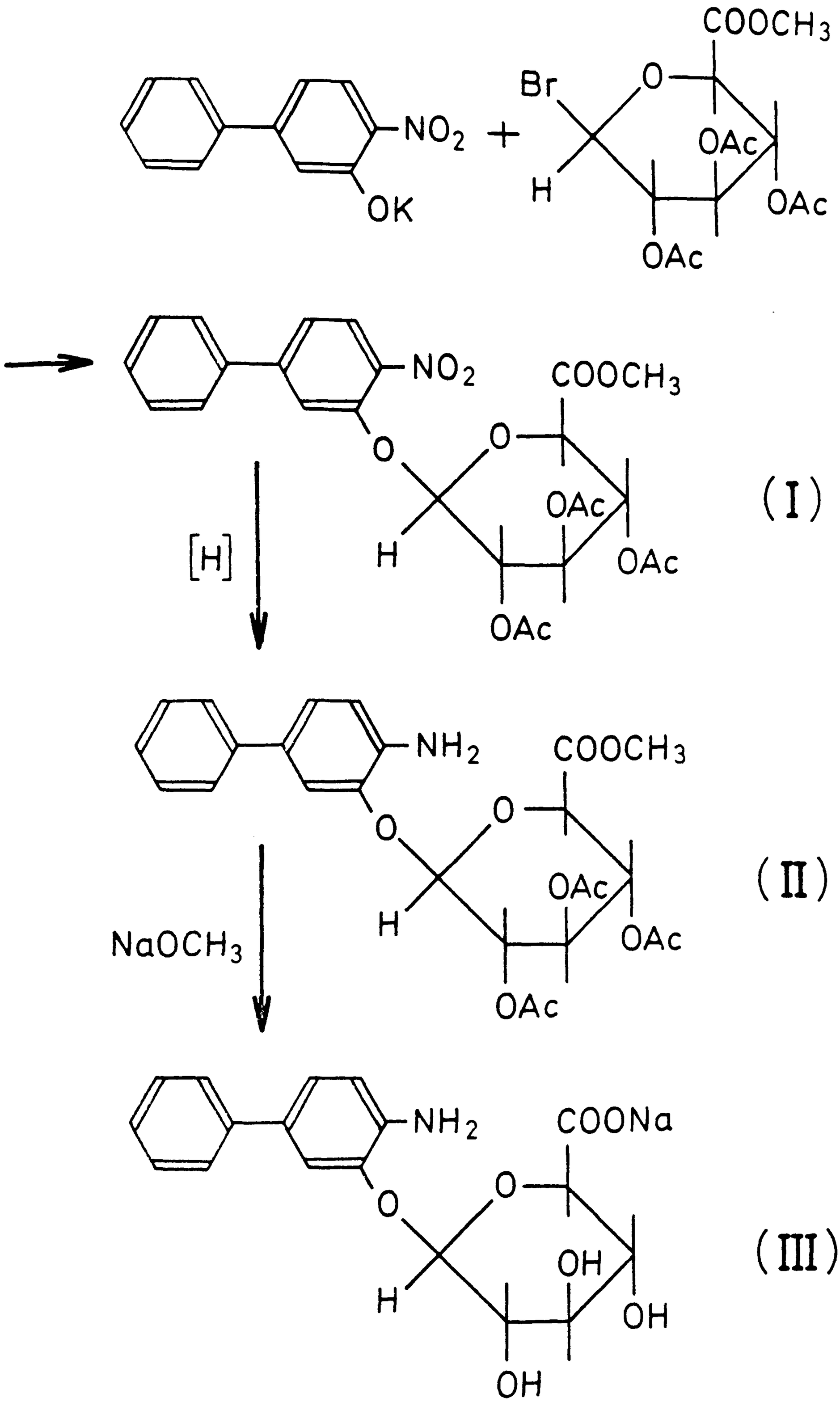
5.3.1 Chemical Synthesis of 4-amino-3-biphenylyl-β-D-glucosiduronic acid

The chemical synthesis of 4-amino-3-biphenylyl-β-D-glucosiduronic acid was attempted by the methods described by Bollenback, Long, Benjamin and Lindquist (1955) via the tri-O-acetylmethylester derivative. Attempts at the direct condensation of methyl tetra-O-acetyl-β-D-glucopyranosiduronate with 4-nitro-3-hydroxybiphenyl, in the presence of para-toluenesulphonic acid as catalyst, at 120°-140°, under a reduced pressure of 12 mm for various times up to two hours, produced only traces of a product with the desired characteristics.

The successful preparation of methyl-(4-nitro-3-biphenylyl-tri-O-acetyl-β-D-glucosid)uronate was accomplished using a modification of the reaction conditions described by Bollenback et al. (1955) for the synthesis of methyl-(O-nitrophenyltri-O-acetyl-β-D-glucopyranosid)uronate.

This synthesis consisted of treating potassium-4-nitro-3-hydroxybiphenylate with methyl tri-O-acetyl-α-D-glucopyranosyl bromide in boiling ethanol. The removal of the acetyl groups and hydrolysis of the methyl ester were unsuccessful when either sodium or barium methoxide were used as described by Bollenback et al. This finding was similar to that of Belman (1961) who observed that hydrolysis of the corresponding compound from 2-nitro-1-naphthol was also unsuccessful. This author decided that prior reduction of the nitro group was a prerequisite for the removal of these protecting groups.

The simple reductive procedure of generating hydrogen from hydrazine hydrate in the presence of a platinum on charcoal catalyst, in situ, in an ethanolic solution of the nitro compound, gave only 4-amino-3-hydroxybiphenyl, indicating that complete hydrolytic cleavage of the carbohydrate moiety had occurred.



(I)

(II)

(III)

Fig.5.3.1. Route used in the synthesis of sodium 4-amino-3-biphenylyl-β-D-glucosiduronate.

Reduction of methyl-(4-nitro-3-biphenylyl-tri-O-acetylglucosid)uronate was accomplished using hydrogen at two atmospheres pressure, at room temperature, in the presence of Adam's platinum oxide catalyst. Treatment of methyl-(4-amino-3-biphenylylglucosid)uronate with barium methoxide gave solutions which rapidly darkened, and did not yield a product with the required properties. However, treatment with sodium methoxide in methanolic solution led to the formation of 4-amino-3-biphenylylglucosiduronic acid.

The synthetic route used in the successful chemical synthesis is outlined in Fig.5.3.1.

5.3.2 Biosynthesis of 4-amino-3-biphenylyl-β-D-glucosiduronic acid

Initially a large scale experiment was carried out using guinea-pig liver microsomes and 4-amino-3-hydroxybiphenyl as the acceptor molecule, in order to confirm that the synthetic and biosynthetic glucuronides had identical properties. This led to the observation that when chromatograms of the biosynthetic products were developed two compounds were detected which gave positive reactions for an aromatic amino group and glucuronic acid. Many experiments were performed, some of which are described, to try to establish the identity of the minor component, but eventually all the evidence indicated that the minor component was the same as the major component, and that splitting during chromatography had been due to inorganic salts present in the concentrate.

Chromatography of the biosynthetic concentrate using solvent systems A or B showed the presence of a major component which had the same R_f values and colour reaction towards spray reagents as the synthetic material. The chromatographic properties are described in Table 5.3.1.

In addition, a minor component was present when developing solvents A or B were used which had identical reactions with the detecting reagents as those of the major component; this trace component was not present when the concentrate was examined using solvent C.

In view of the fact that the presence of a second compound could invalidate any investigation of the species difference in the formation of 4-amino-3-biphenylylglucosiduronic acid, some further experiments were carried out in the hope of gaining further information as to the nature of the minor component. That only one substance was detected using solvent system C suggested that a labile complex with some constituent of the incubation medium was formed, or possibly an N-glucuronide. However, the addition of some synthetic 4-amino-3-biphenylylglucosiduronic acid to either the incubation medium, a solution of glucuronic acid or a solution of ascorbic acid failed to produce a complex with the properties of the trace component.

Two further possibilities were then examined, the first that a glucoside had been formed due to the presence of uridine diphosphoglucose (UDPG) as an impurity in the UDPGA, and the second that the uridine diphosphate (UDP) formed by the action of the glucuronyl transferase could act as a phosphorylating agent.

In order to test these ideas, experiments were carried out as described in 5.2.4, except that either UDPG or UDP were substituted for UDPGA. Synthetic 4-amino-3-biphenylylgluconiduronic acid was also examined as an acceptor molecule. All these experiments failed to produce evidence for the nature of the minor component.

The discovery of facile migration reactions as intermediates in metabolism suggested a remote possibility that migration of the hydroxyl group either before or after conjugation with glucuronic acid could occur. Further experiments were then performed in which 4-amino-4'-hydroxybiphenyl was used as an acceptor molecule in place of 4-amino-3-hydroxybiphenyl in the system described in 5.2.4.

A conjugate with properties suggesting that it was the O-glucosiduronic acid was formed, the chromatographic properties of this compound being different from those of the "minor" component.

A further experiment in which phosphate was completely eliminated from the reaction mixture by replacing the phosphate buffer with 0.1M tris pH 7.4 indicated that the presence of traces of inorganic material could significantly alter the R_f of 4-amino-3-biphenylylglucosiduronic acid. The addition of various salts to samples of synthetic 4-amino-3-biphenylylglucosiduronic acid, followed by chromatographic examination, showed that the minor component was formed due to the presence of certain salts. This was particularly pronounced in the case of ammonium acetate, but occurred also in the presence of disodium hydrogen phosphate.

Elution of the minor component from sheets of 3MM chromatography paper with water or methanol containing ammonia, followed by concentration using a rotary film evaporator at 40°, gave only a substance having the properties of the major component when re-examined by chromatography using solvent systems A or B.

From the foregoing it has to be concluded that when 4-amino-3-hydroxybiphenyl is incubated in the presence of microsomes and UDPGA only one

substance is formed, although due to the presence of certain salts complexes may be formed with slightly different chromatographic properties.

5.3.3 Comparison of the synthetic and biosynthetic 4-amino-3-biphenylyl-glucosiduronic acid.

The synthetic and biosynthetic 4-amino-3-biphenylylglucosiduronic acids had identical chromatographic properties and response to detection reagents when examined by paper chromatography (Table 5.3.1). When treated with nitrous acid followed by NED, both synthetic and biosynthetic materials gave rise to azo-dyes having identical absorption spectra with a maximum at 572 nm (Fig.5.3.2). Both synthetic and biosynthetic 4-amino-3-biphenylylglucosiduronic acids gave a positive reaction with Tollen's naphthoresorcinol reagent, and were hydrolysed by bacterial and mammalian β-glucuronidase. The enzymic hydrolysis was inhibited by the incorporation of glucosaccharo-1:4- lactone into the incubate. Chromatographic examination indicated the disappearance of the substance having the chromatographic properties of 4-amino-3-biphenylyl-glucosiduronic acid under the influence of β-glucuronidase.

It is concluded that the synthetic and biosynthetic materials prepared have identical properties which are those expected of a glucuronic acid conjugate of an ortho-aminophenol .

5.3.4 Development of assay and species variation of uridine diphosphoglucuronic acid : 4-amino-3-hydroxybiphenylglucuronyl transferase.

The finding that 4-amino-3-hydroxybiphenyl gave rise to only one substance when incubated with UDPGA in the presence of guinea-pig hepatic microsomes, and that this substance was identical in all respects with 4-amino-3-biphenylyl glucosiduronate, allowed the development of an assay for the enzyme transferring

Table 5.3.1. Chromatographic properties* and response to detecting reagents shown by synthetic and biosynthetic 4-amino-3-biphenylyl-glucosiduronate.

Detecting Reagent	Response	
	Synthetic	Biosynthetic
nitrous acid followed by alkaline		
(a) 1-naphthol	orange-pink	orange-pink
(b) 2-naphthol	red	red
(c) hexylresorcinol	yellow	yellow
p-dimethylaminobenzaldehyde	yellow	yellow
p-dimethylaminocinnamaldehyde	magenta	magenta
naphthoresorcinol in TCA+ at 100°	pink	pink
potassium dichromate-silver nitrate	yellow	yellow
ultra-violet light	blue	blue

Solvent	Synthetic Rf	Biosynthetic Rf
Butan-1-ol satd. with 2N Ammonia	0.2	0.2
Butan-1-ol, Propan-1-ol, 2N Ammonia 2:1:1 v/v	0.23	0.23
Butan-1-ol, Acetic acid, Water 12:3:5 v/v	0.62	0.62

* Descending on Whatman No.1 paper at room temperature.

\+ Trichloracetic acid.

the glucuronic acid moiety from UDPGA to 4-amino-3-hydroxybiphenyl. The failure of ortho-aminophenols to diazotise and couple, due to the formation of diazo-oxides, has previously been utilised by Dutton & Storey (1962) to allow the measurement of ortho-aminophenylglucuronide in the presence of ortho-aminophenol by a method involving diazotisation and coupling with NED. The resulting coloured azo compound was measured and the intensity of colour shown to be proportional to the amount of ortho-aminophenylglucuronide formed. This method was adapted for the measurement of 4-amino-3-biphenylylglucuronide.

5.3.4.1 Effect of Temperature and Time on Colour Development.

Solutions of synthetic 4-amino-3-biphenylylglucosiduronate, (Ca. 4 μmoles/ml), were treated successively with 2N hydrochloric acid (1 ml), sodium nitrite 0.05% (1 ml), ammonium sulphamate 0.5% (1 ml) and NED 0.1% (1 ml). The reaction mixtures were allowed to stand at room temperature for three minutes after the addition of sodium nitrite and again after the addition of the ammonium sulphamate. Immediately after the addition of the NED, the reaction mixtures were placed in water-baths at either 37° or 65°.

The absorbtion was measured at 572 nm in 1 cm glass cells at various time intervals and the results are shown in Fig.5.3.3. From these results it can be clearly seen that development of the colour at 65° results in maximum absorbtion in 30 min, whereas at 37° it takes at least 60 min to obtain the same colour intensity. It was decided that the colour would be developed at 65° for 30 min in all subsequent experiments.

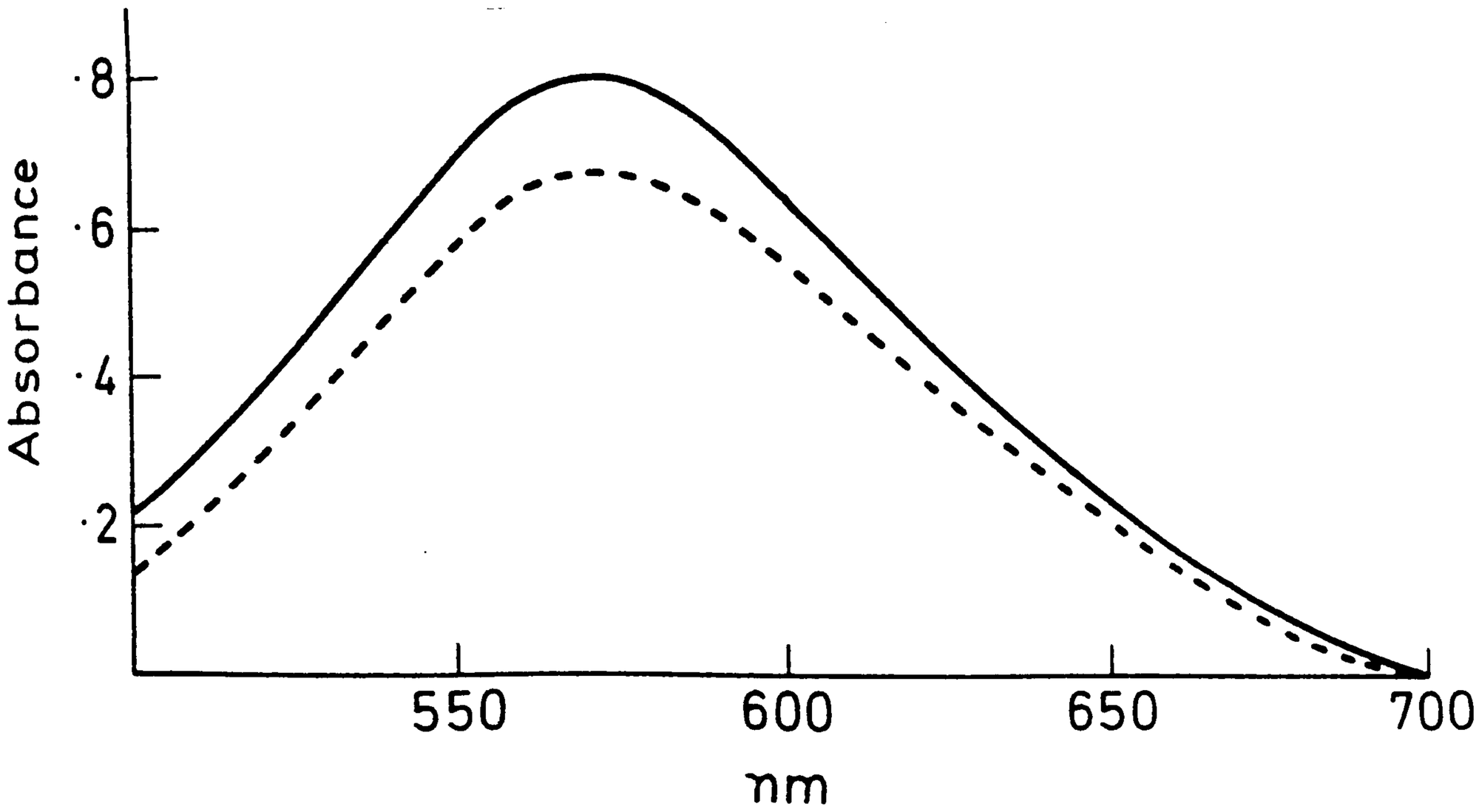


Fig.5.3.2. Comparison of azo dye spectra derived from synthetic and biosynthetic 4-amino-3-biphenylylglucuronide.
---- Synthetic (0.2 μM/ml). —— Biosynthetic (rabbit liver microsomes).

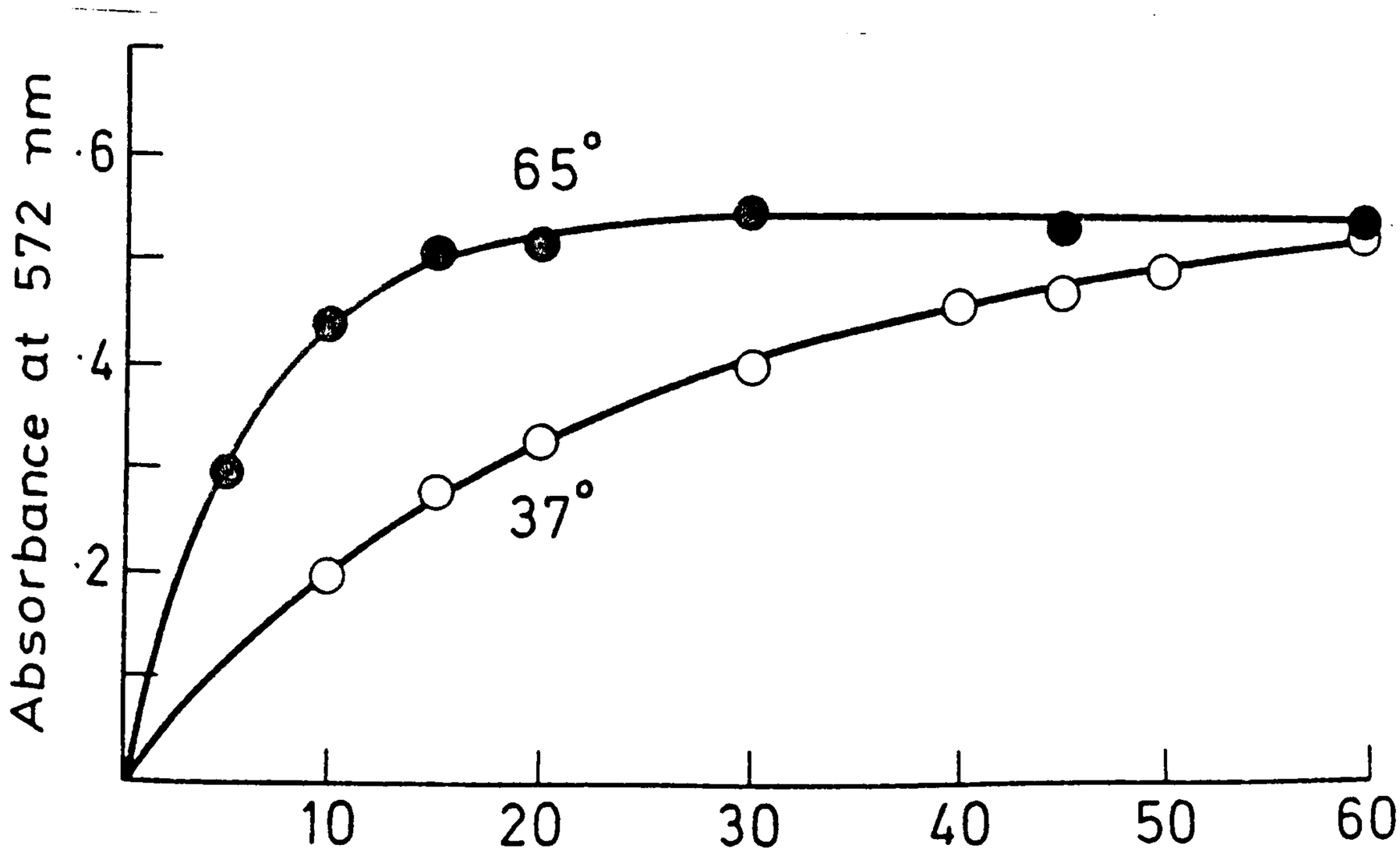


Fig.5.3.3. Time Course of Colour development at 37 and 65° following diazotisation of 4-amino-3-biphenylylglucuronide and coupling with NED.

5.3.4.2 Effect of Trichloracetic acid concentration on subsequent colour development

Synthetic 4-amino-3-biphenylylglucuronide solution, ca. 4 μmoles in 1 ml of 0.2% ascorbic acid, was added to rabbit liver microsomes prepared from 0.5 g of liver resuspended in 2 ml of phosphate buffer pH 7.4. The microsomal suspension was treated with 1 ml of trichloracetic acid solution and centrifuged at 10,000xg for 10 min. The experiment was repeated using concentrations of trichloracetic acid from 3.5% w/v to 10% w/v. Aliquots (3 ml) of the supernatant were treated with sodium nitrite, ammonium sulphamate and NED as described in 5.3.4.1. The absorbance was measured at 572 nm and the results are shown in Table 5.3.2.

Table 5.3.2. Effect of trichloracetic acid concentration on subsequent colour development

% Trichloracetic Acid	Absorbance (572 nm)
3.5	0.28
4.0	0.36
4.5	0.41
5.0	0.37
7.5	0.41
10.0	0.41

It was decided that 7.5% trichloracetic acid would be used as a protein precipitant in the glucuronyl transferase assay.

5.3.4.3. Linearity of absorbance versus concentration in the assay of 4-amino-3-biphenylylglucosiduronic acid

Various concentrations of synthetic 4-amino-3-biphenylylglucosiduronic acid were incorporated into mixtures of microsomal tissue plus UDPGA, treated with trichloracetic acid(7.5%), centrifuged, and aliquots of the supernatant treated with sodium nitrite, ammonium sulphamate and NED, as in 5.3.4.1. The reaction is linear over the range 0.02-0.5 μmoles/flask (Fig.5.3.4).

5.3.4.4. Effect of 4-amino-3-hydroxybiphenyl concentration on enzyme activity

Guinea-pig liver microsomes from 0.5 g liver, resuspended in 0.5 ml isotonic potassium chloride, were incubated with UDPGA (final concentration 2 mM) in phosphate buffer pH 7.4 containing magnesium chloride 0.02M (0.5 ml) and 4-amino-3-hydroxybiphenyl in 0.2% ascorbic acid (1.0 ml). The effect of 4-amino-3-hydroxybiphenyl, ranging from 0.1 μ mole to 1.2 μmole per 2 ml incubate, on the amount of conjugate formed was examined.

The colour development was carried out as described in 5.3.4.1 and 5.3.4.2. The results are shown in Fig.5.3.5. From these results it was decided to use a final concentration of 0.3 mM (equivalent to 0.6 μmoles per flask) for all further experiments.

5.3.4.5. Final assay procedure for 4-amino-3-hydroxybiphenyl: UDPGA glucuronyl transferase.

From the foregoing experiments described in this section the following assay for enzymic activity was developed. This assay was used in the subsequent investigation of the glucuronyl transferase activity present in the hepatic microsomes of various animal species.

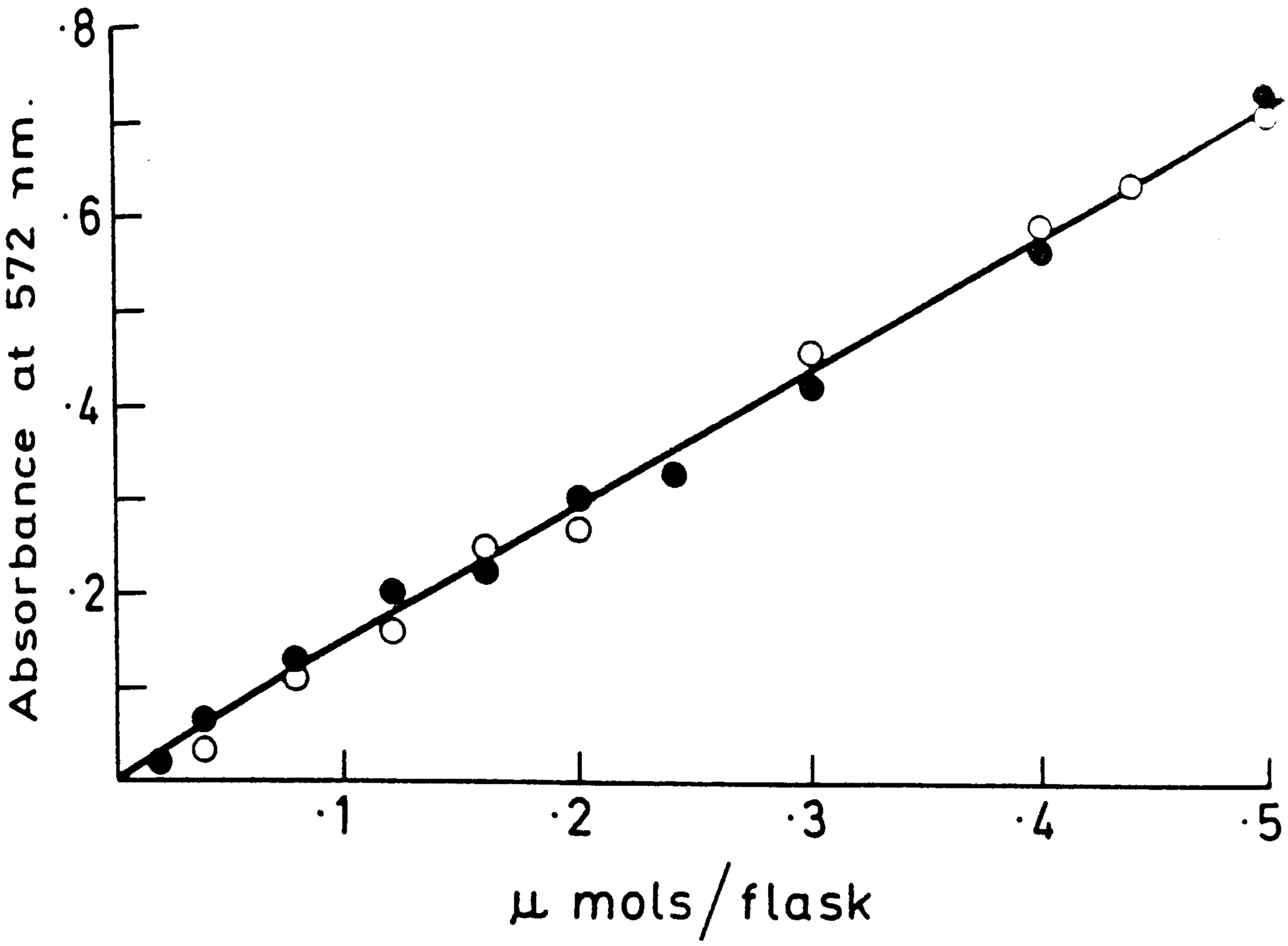


Fig.5.3.4. Linearity of assay for 4-amino-3-biphenylylglucuronic acid

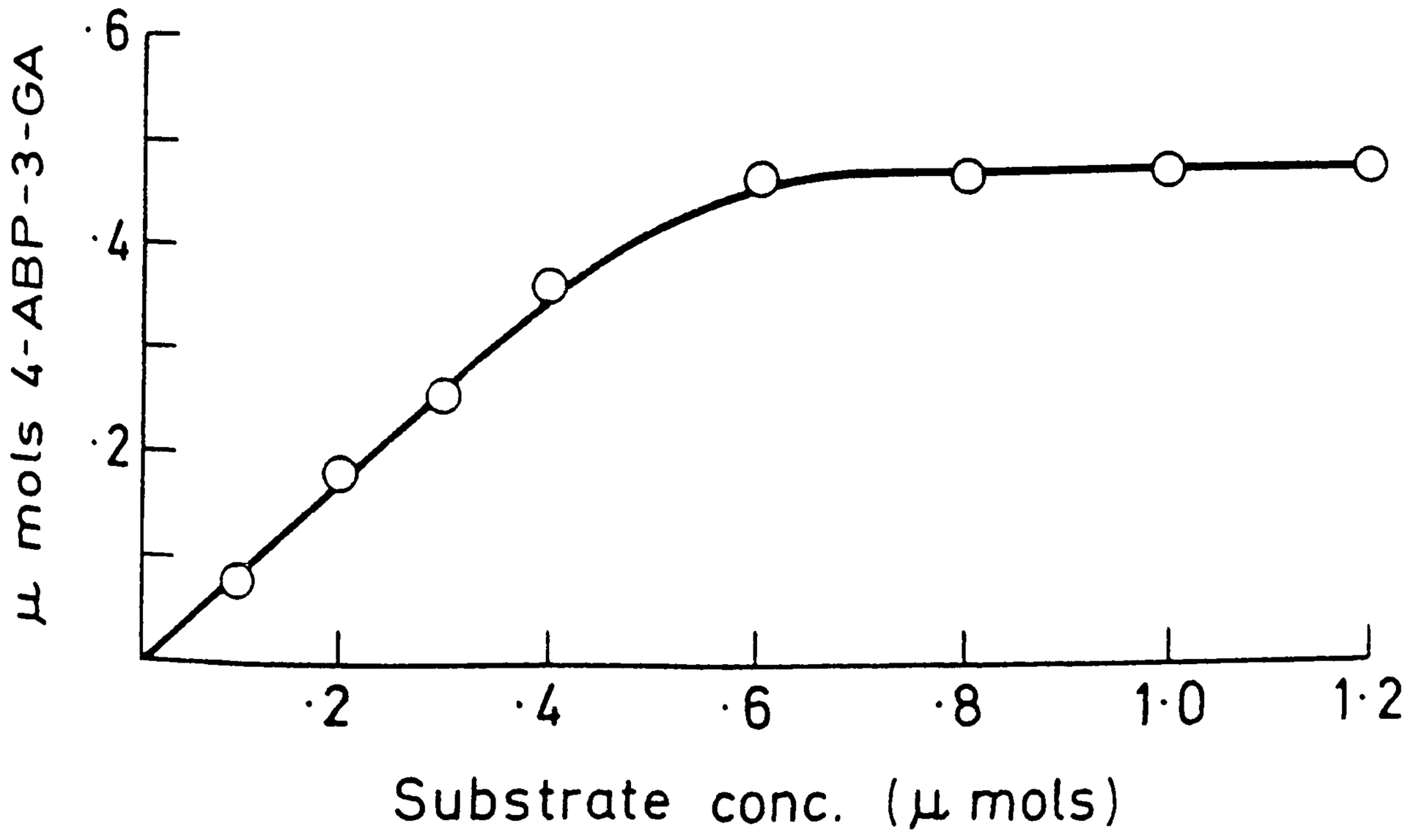


Fig. 5.3.5. Effect of 4-amino-3-hydroxybiphenyl concentration on 4-amino-3-hydroxybiphenyl: UDPGA glucuronyl transferase

4-ABP-3-GA = 4-amino-3-biphenylyl glucuronic acid

a) Incubation medium

Microsomes from 0.5g of liver resuspended in 1.15% KCl 0.5 ml.

Uridine diphosphoglucuronic acid (10 mg) dissolved in phosphate buffer pH 7.4 containing 0.02M magnesium chloride 1.0 ml.

4-Amino-3-hydroxybiphenyl (0.6 μmoles in 0.2% ascorbic acid). 0.5 ml.

b) Incubation conditions

The enzyme preparations were incubated at 37° in 25 ml narrow-necked conical flasks for 30 m unless otherwise stated.

c) Colour development

The reaction was terminated by the addition of trichloracetic acid 7.5% w/v (1.0 ml), and the protein sedimented by centrifugation at 10,000xg for 10 m Aliquots of the supernatants (2.0 ml) were transferred to 5 ml stoppered test tubes and successively treated with sodium nitrite solution 0.05% (1.0 ml), ammonium sulphamate solution 0.5% (1.0 ml), and NED 0.1% (1.0 ml). The tubes were allowed to stand at room temperature for 3 m after the addition of the sodium nitrite, and again for 3 m after the addition of the ammonium sulphamate.

Immediately after the addition of the NED the tubes were stoppered and placed in a water-bath at 65° for 30 m.

After cooling to room temperature the absorbance was read against a blank, prepared exactly as described above but without the addition of UDPGA to the initial incubation mixture.

5.3.4.6 Effect of time on the course of the biosynthesis of 4-amino-3-biphenylyl-glucosiduronic acid.

Under the conditions of assay developed (5.3.4.5), the time course of the biosynthesis of 4-amino-3-biphenylylglucosiduronic acid was determined for a rabbit

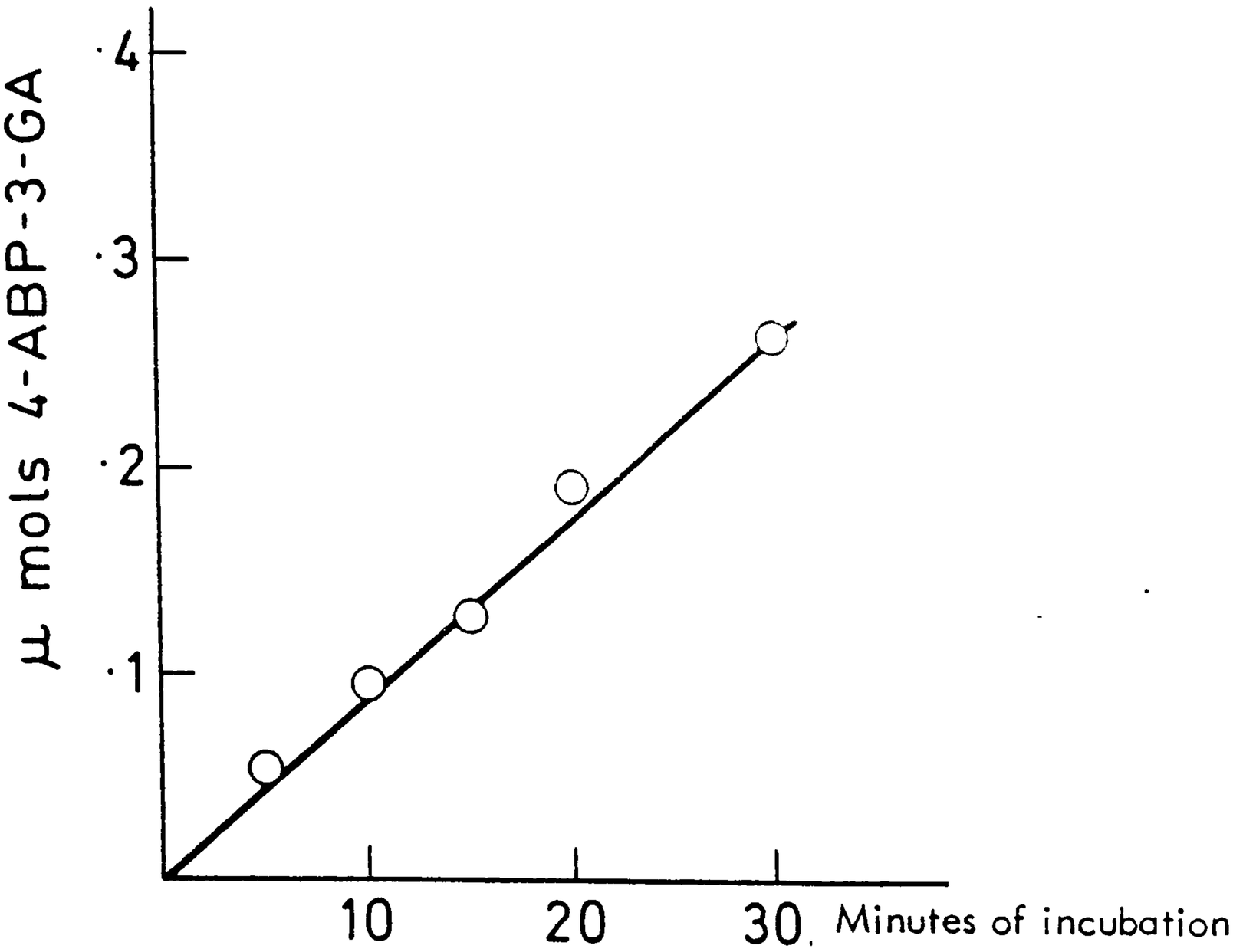
hepatic microsomal preparation. The results are shown in Fig.5.3.6 where it can be seen that the biosynthesis is linear, at least up to thirty minutes. This incubation time was therefore used as the standard incubation time for the species variation experiments.

5.3.4.7. Species variation in the biosynthesis of 4-amino-3-biphenylylglucosid-uronic acid.

The glucuronyl transferase activity towards 4-amino-3-hydroxybiphenyl was determined under the assay conditions described in 5.3.4.5 for hepatic microsomal preparations from various animal species, during the course of 30 m incubation. The results are presented in Table 5.3.3 and indicate that considerable species variation in the ability to form "glucuronides" does exist. It is well documented that the cat lacks the ability to form these conjugates (Dutton, 1966), and the finding that human foetal tissue also did not produce measurable amounts of conjugate is in line with the low levels of glucuronyl transferase usually associated with the neonate (See Section 6).

5.3.5. The urinary excretion of 4-amino-3-biphenylylglucosiduronic acid by various animal species.

Following the i.p. administration of 4-amino-3-hydroxybiphenyl to the rat, cat and guinea-pig, the urine collected from these species was examined by paper chromatography as described earlier. In all species studied only two substances were detected which had an aromatic aminogroup as shown by specific spray detection reagents, although many other compounds could be seen using ultra-violet light. In the case of the guinea-pig the principal substance detected was identical in every respect with 4-amino-3-biphenylylglucosiduronic acid. The minor metabolite had properties identical to those of an authentic sample of



4-ABP-3-GA = 4-amino-3-biphenylyl glucuronic acid

Fig.5.3.6 Linearity of rabbit microsomal 4-amino-3-hydroxybiphenyl:UDPGA glucuronyl transferase with time of incubation.

Table 5.3.3. Uridine diphosphoglucuronic acid : 4-amino-3-hydroxybiphenyl glucuronyl transferase in various species

Species	Activity *	
	Expt.1	Expt.2
Guinea-pig	0.44	0.38
Rabbit	0.37	0.34
Dog	0.37	0.31
Hamster	0.30	0.28
Mouse	0.25	0.30
Rat	0.20	0.18
Cat	0.05	0.02
Human Foetus	0	0

* Results are expressed as μmoles 4ABPGA formed per 0.5g liver during 30 m incubation at 37°.

4-amino-3-biphenylylsulphate (gift from Dr.P.Sims). The urine obtained from the rat contained 4-amino-3-biphenylylsulphate as the principal detected metabolite, the glucosiduronic acid conjugate being present to a slightly smaller extent as judged from visual examination of the chromatograms. In the cat no evidence was found to indicate that this species could form a glucuronic acid metabolite; the only substance detected with an aromatic amino group had properties identical to those of 4-amino-3-biphenylyl sulphate.

The results presented above are in line with the in vitro results shown in Table 5.3.3. The high level of this glucuronyl transferase in the guinea-pig is reflected in the finding of the glucuronide as the principal metabolite from 4-amino-3-hydroxybiphenyl. Conversely, the very low level of glucuronyl transferase activity found in vitro in the cat hepatic preparations would indicate the cat's relative inability to utilise this metabolic pathway. The finding of medium levels of the glucuronyl transferase in rat liver preparations is consistent with the detection of approximately equal amounts of both the sulphate and glucuronic acid conjugates.

Clearly no direct correlation can be obtained between the UDPGA:4-amino-3-hydroxybiphenyl glucuronyl transferase and carcinogenic activity of 4-amino-biphenyl in the species studied. It is of interest that the species usually considered refractory to the carcinogenic effect of aromatic amines, i.e. the guinea-pig, possesses the highest glucuronyl transferase activity towards 4-amino-3-hydroxybiphenyl of those species studied. In comparison, the dog, which is the species most susceptible to bladder carcinogenesis by aromatic amines, had only a slightly lower glucuronyltransferase activity. The low

activity of glucuronyl transferase demonstrated in the cat may, in part, play a role in the high acute toxicity of aromatic amines associated with this species (Rohde, 1923). It would seem that the other species examined do have sufficient levels of the glucuronyl transferase to allow conjugation of 4-amino-3-hydroxybiphenyl if presented with it as substrate. The results in Section 2 indicate that the levels of ortho-hydroxylation of 4-aminobiphenyl in any species is likely to depend on the ability of that species to acetylate 4-aminobiphenyl and deacetylate 4-acetamidobiphenyl. If the correct balance occurs and 4-amino-3-hydroxybiphenyl is formed, the results obtained indicate that sufficient glucuronyl transferase is present to allow the formation and excretion of the glucuronide conjugate. The finding that this conjugate is hydrolysed by β-glucuronidase is in agreement with the original hypothesis proposed by Boyland (1956). The lack of a correlation between glucuronyl transferase activity and susceptibility towards amine carcinogenicity found in this study in no way invalidates Boyland's hypothesis.

SECTION 6 CARCINOGENICITY OF SOME HYDROXYLATED DERIVATIVES OF 4-AMINOBIPHENYL

6.1 INTRODUCTION

4-Aminobiphenyl or compounds which are metabolised to this amine 'in vivo' e.g. 4-nitrobiphenyl and 4-acetamidobiphenyl are powerful carcinogens in a variety of species. In man (Melick et al. 1955), dog (Walpole et al. 1954) and rabbits (Bonser, 1962; Wood, 1970), 4-aminobiphenyl induces cancer of the urinary tract, whereas in rats (Walpole et al. 1952) it gives rise to both intestinal and liver tumours. After the administration of 4-aminobiphenyl by stomach tube to AB x IF mice, no alteration of spontaneous hepatoma incidence was found by Clayson, Lawson, Santana & Bonser (1965); however, two carcinomas of the urinary bladder were found in twelve surviving animals. In a later paper, Clayson, Lawson & Pringle (1967) found that this compound produced a high yield of hepatomas in female C57 x IF mice, but no urinary bladder tumours. It appears that even in species which are susceptible to the action of 4-aminobiphenyl, multiple daily dosing for long periods may be necessary to produce tumours. Deichmann & McDonald (1968) found that massive single doses of 4-aminobiphenyl given to dogs failed to produce any carcinogenic response.

Because of its structural similarity to the known carcinogen 2-acetamido-fluorene, 4-acetamidobiphenyl was tested for carcinogenicity in rats by Miller, Sandin, Miller & Rusch (1956) and was shown to be a mammary carcinogen with no effect on the urinary bladder. This compound, when fed to female dogs, however, produced tumours of the bladder with pulmonary metastases (Jabara, 1963). Deichmann et al. (1958) showed that 4-nitrobiphenyl was carcinogenic to the dog urinary bladder, and Laham et al. (1964) showed that mammary tumours were produced with this compound when given to female rats.

However, despite the extensive studies on the metabolism of these compounds, as discussed in Section 2, the routes leading to the initiation of the carcinogenic response remain obscure. As a carcinogenic response to all three compounds has been found in the dog, and as interconversion to 4-aminobiphenyl, but not to 4-acetamidobiphenyl or 4-nitrobiphenyl, would be expected, it is not inconceivable that a metabolite derived from 4-aminobiphenyl is the responsible agent.

Three hydroxylated metabolites derived from 4-aminobiphenyl have been reported, viz., 4-amino-3-hydroxybiphenyl, 4-amino-4'-hydroxybiphenyl and 4-hydroxylaminobiphenyl. (Section 2). However, knowledge as to their carcinogenicity is poor and conflicting. When 4-amino-3-hydroxybiphenyl was incorporated into pellets and implanted into the urinary bladders of mice, it proved to be carcinogenic (Clayson et al. 1958). Using similar bladder implantation testing conditions, 4-aminobiphenyl, 4-amino-4'-hydroxybiphenyl and 4-hydroxylaminobiphenyl, were all shown to be inactive (Bonser et al. 1963; Clayson et al. 1958; Boyland et al. 1964). In the latter report 4-N-acetyl-N-hydroxylaminobiphenyl was also shown to be inactive in the mouse bladder implantation tests.

When 4-amino-3-hydroxybiphenyl was incorporated into the diets of rats (Miller et al, 1956) it was inactive, whereas the N-acetyl derivative of 4-hydroxylaminobiphenyl was active in this test system (Miller, Wyatt, Miller & Hartmann, 1961), producing high yields of mammary tumours.

Because of the conflicting evidence as to the nature of the proximate carcinogen derived from 4-aminobiphenyl it seemed desirable to examine this compound and its metabolites in a further test system.

Pietra, Spencer and Shubik (1959) showed that injection of mice with only 30 μg of 9,10-dimethyl-1,2-benzanthracene during the first day of life, gave

rise to high yields of lung adenomas and malignant lymphomas. This method of testing substances for carcinogenic activity rapidly gained support and a large number of compounds which had previously required multiple dosing or life-span experiments gave positive results after one to three doses during the neonatal period.

Roe, Rowson and Salaman (1961) showed that tumours of many sites could be elicited using this test system, depending upon the nature of the toxic agent, and recognised that this was an extremely sensitive test for carcinogenesis studies. This sensitivity is probably due to many factors including the possible role of viral infection, the lack of full immunological competence and the hormonal status of the test animal. These factors are known to modify the response to carcinogens in adult animals and may play some role in the neonate.

It is known that young animals either lack or have decreased levels of many microsomal drug-hydroxylating enzymes (Jondorf, Maickel & Brodie, 1958; Fouts & Adamson, 1959). In addition the ability to synthesise certain glucosiduronates and other conjugates is slow to develop in animals and man (Dutton, 1963; Vest, 1965; Vest & Salzberg, 1965). This would indicate that after injection of a substance to newborn mice, further biotransformation would be minimal, and exposure to the substance administered would be sustained. Evidence that this occurs has been obtained with the compound ethylcarbamate. With this compound, total elimination of a single dose of 0.5 mg/g took only eight hours in adult mice, whereas three days were required in newborn mice (Kaye, 1960; Mirvish, Cividalli & Berenblum, 1964).

Similarly, Domsky, Lijinsky, Spencer & Shubik (1963) showed that only 10% of a dose of 7,12-dimethylbenz (a) anthracene remained in the adult mouse four days after dosing; in the newborn mouse however, no decrease in the level of the compound administered was found even ten days after injection.

It was therefore thought that the new-born mouse would be a suitable test system to indicate which, if any, of the known hydroxylated metabolites of 4-aminobiphenyl were likely proximate carcinogens.

6.2 EXPERIMENTAL

6.2.1 Chemicals

4-Aminobiphenyl, 4-hydroxylaminobiphenyl, 4-amino-3-hydroxybiphenyl and 4-amino-4'-hydroxybiphenyl were all prepared and purified as described in Section 2.

7,12-Dimethylbenz (a) anthracene (DMBA), which was used as a positive control, was obtained from Koch-Light Laboratories.

6.2.2 Injection Media

All the test compounds were suspended by ultrasonication in 3% aqueous gelatin for injection.

6.2.3 Animals

Four hundred and sixty newborn Swiss (Porton) strain mice were used. The animals were obtained from a caesarean-derived strain maintained under barrier conditions. The animals were randomised, within 24 hours of birth, into seven experimental groups. With the exception of a control group, each

group received one of the compounds or aqueous gelatine. After the first injection each mouse was returned to a mother, each of whom was given ten neonates.

6.2.4 Administration of Test Substances.

The mice were injected subcutaneously in the interscapular region on each of the first three days of life. They were treated as follows:

Group I : Three injections of 200 μg 4-aminobiphenyl in 0.02 ml 3% aqueous gelatin

Group II : Three injections of 200 μg 4-amino-3-hydroxybiphenyl in 0.02 ml 3% aqueous gelatin

Group III : Three injections of 200 μg 4-hydroxylaminobiphenyl in 0.02 ml 3% aqueous gelatin

Group IV : Three injections of 200 μg 4-amino-4'-hydroxybiphenyl in 0.02 ml 3% aqueous gelatin

Group V : Three injections of 0.02 ml aqueous gelatin

Group VI : No injections.

Group VII : Three injections of 20 μg DMBA in 0.02 ml 3% aqueous gelatin

Groups I-IV constitute the test groups, groups V-VII being control groups. The latter group VII was included to demonstrate that the strain of mice used was sensitive to a known carcinogen.

6.2.5 Conduct of experiment.

The mice were weaned at four weeks and the sexes segregated. They were then housed in plastic cages, ten mice in each, fed an autoclaved cubed diet (Small Animal Diet, Spillers Ltd.), and given water ad libitum. Barrier

conditions were maintained and all bedding was sterilised. The mice were inspected daily and thoroughly examined daily. Sick animals were killed promptly and all survivors were killed between forty-eight and fifty-two weeks after birth.

6.2.6 Postmortem examinations

Full postmortem examinations were carried out on all mice. The liver, kidneys and urinary bladder were removed routinely, together with other organs that showed any abnormalities, and fixed in Bouin's solution. Paraffin sections were prepared at 5μ, stained with hematoxylin and eosin and where necessary with hematoxylin and van Gieson, Mason's trichrome, Gordon and Sweet's silver impregnation method for reticulin and periodic acid-Schiff.

6.3 RESULTS AND DISCUSSION

The numbers of mice treated in each group, the number alive at weaning, and the number of mice alive at forty-eight to fifty-two weeks are recorded in Table 6.3.1. The distribution of the sexes in the groups is also shown. Survival rates in all groups was very good, always exceeding seventy per cent of those alive at weaning. In most cases over ninety per cent of the animals survived and there was no evidence to suggest that either sex was more sensitive to the treatments. The principal neoplasms seen in these experimental animals were hepatomas and the distribution of these is shown in Fig.6.3.1. The incidence of liver tumours which occurred in the untreated group VI was very low and occurred exclusively in the male animals. The group treated with aqueous gelatin alone (Group V) had a slightly raised incidence of hepatomas, although this observation was of doubtful significance.

Table 6.3.1. Survival of mice in Groups I-VII

Experimental groups	Number of mice given injections at < 24 hrs	Number of mice alive at weaning	Number of mice alive at 48-52 weeks	Survivors at 48-52 weeks as % of those alive at weaning.
Group I: 4-Aminobiphenyl	52	♂ 24 51 ♀ 27	♂ 20 43 ♀ 23	♂ 85.4 ♀ 92.0
Group II: 4-Amino-3-hydroxybiphenyl	55	♂ 23 55 ♀ 32	♂ 19 48 ♀ 29	♂ 82.6 ♀ 90.6
Group III: 4-Hydroxylamino-biphenyl	56	♂ 20 55 ♀ 35	♂ 19 52 ♀ 33	♂ 95.0 ♀ 94.3
Group IV: 4-Amino-4'-hydroxybiphenyl	50	♂ 22 49 ♀ 27	♂ 18 44 ♀ 26	♂ 81.8 ♀ 96.3
Group V: Aqueous gelatin	100	♂ 48 98 ♀ 50	♂ 41 87 ♀ 46	♂ 85.4 ♀ 92.0
Group VI: Untreated	98	♂ 45 96 ♀ 51	♂ 42 90 ♀ 48	♂ 93.3 ♀ 94.1
Group VII: 7,12-Dimethyl-benz(a)anthracene	49	♂ 26 49 ♀ 23	♂ 19 39 ♀ 20	♂ 73.1 ♀ 87.0

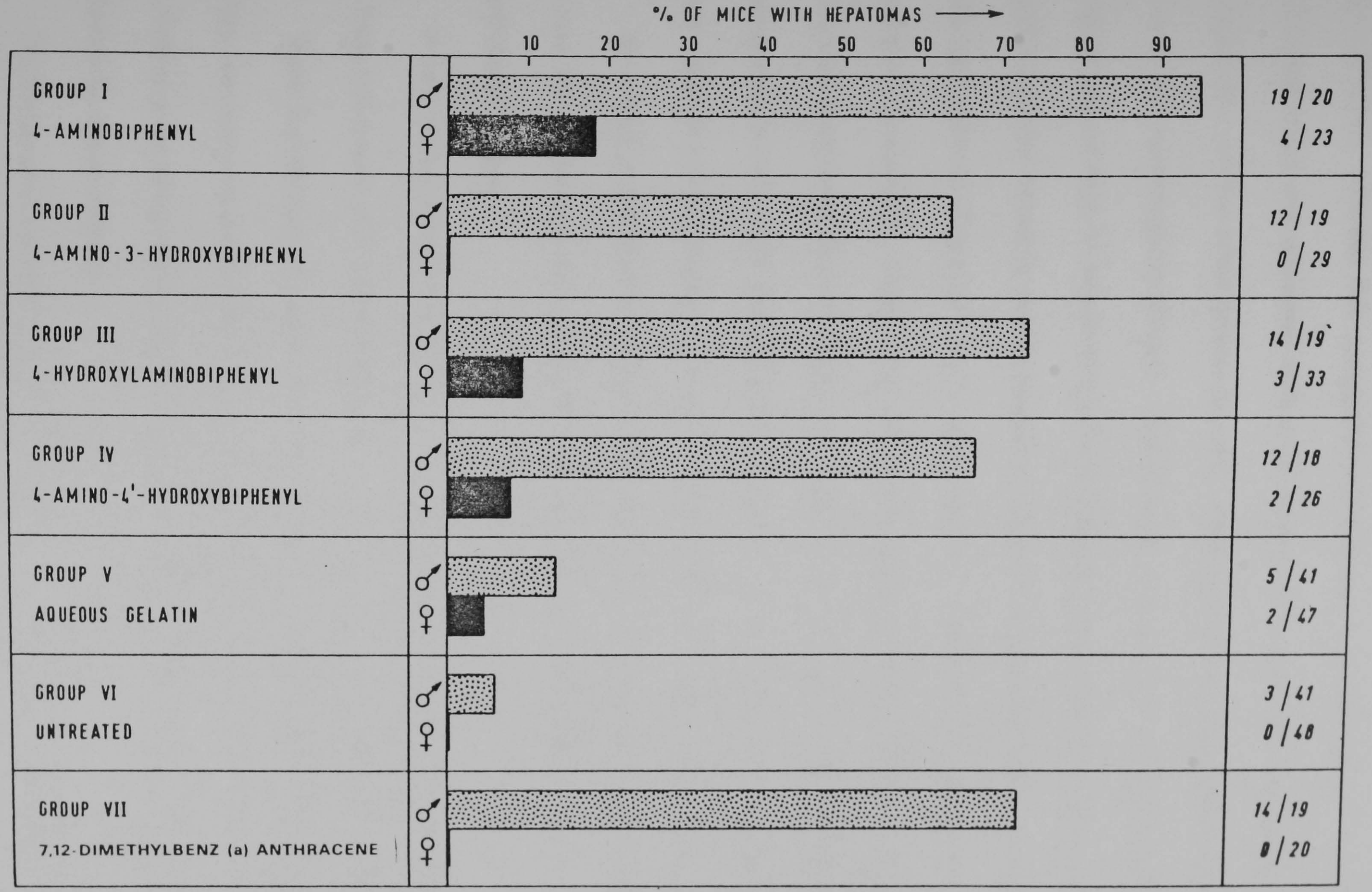


Fig. 6.3.1 Incidence of hepatomas in mice of groups I to VII killed at termination of experiment (48 - 52 weeks)

In the group treated with DMBA high levels of hepatomas were produced in male mice, whereas this compound was inactive in female mice. An almost identical result was obtained with mice treated with 4-amino-3-hydroxybiphenyl (Group II). In the other groups as well, male mice were always the most sensitive to the carcinogenic stimuli. Surprisingly, the parent amine produced the highest percentage of hepatomas,with 4-hydroxylaminobiphenyl slightly less active and the isomeric aminophenols equipotent in the male mice. In most affected animals of groups I to IV and group VII multiple liver tumours were present. In contrast, those mice affected in groups V and VI never had more than two hepatomas found in any one animal. In view of the low incidence of hepatomas found in the female mice of groups II, V, VI and VII, it is probable that those found in groups I, III and IV were produced by the test compounds.

As a high proportion of animals survived the full duration of the experiment it was not possible to determine the time of appearance of hepatomas in individual groups.

A low incidence of other tumours was seen in groups I-VI and, as expected, a high incidence of pulmonary adenoma was found in animals treated with DMBA.

These incidences of tumours are recorded in Table 6.3.2 and show a surprisingly low occurrence of pulmonary adenoma in those animals treated with amines.

A further surprising result was the complete absence of tumours of the urinary bladder in these animals.

The histopathological examination carried out by Drs. Carter and Roe is reported as follows:-

Table 6.3.2. Incidence of tumors other than hepatomas

Experimental group	Pulmonary adenoma	Thymic lymphoma	Lymphosarcoma of spleen	Generalised malignant lymphoma	Other tumours
Group I: 4-Aminobiphenyl	1	1	1	-	-
Group II: 4-Amino-3-hydroxybiphenyl	2	-	-	-	-
Group III: 4-Hydroxylaminobiphenyl	-	-	-	1	-
Group IV: 4-Amino-4'-hydroxybiphenyl	1	-	-	1	-
Group V: Aqueous gelatin	1	1	-	-	-
Group VI: Untreated	2	-	1	-	-
Group VII: 7,12-Dimethylbenz(a)anthracene	29	3	-	4	1 granulosa-cell tumour of ovary

"Liver: The macroscopic and microscopic appearances of the hepatomas were similar in all experimental groups. The lesions were usually multiple and showed no predilection for any particular zone of the liver. They varied in size, ranging from small nodules to large, protruding masses measuring up to 2.5-3.0 cm in diameter. The predominant cellular pattern was of well-differentiated cords, interspersed with large, blood-filled spaces. Hyaline cytoplasmic inclusions were seen in several tumours from all experimental groups. No bile-duct elements were identified. The tumours often showed patchy necrosis or fatty degeneration, and such changes were not necessarily accompanied by parallel abnormalities in the surrounding non-neoplastic parenchyma. Foci of haemorrhage and necrosis were sometimes prominent and some tumours showed extensive infarction. The surrounding parenchyma was often compressed but no extension of tumours into or beyond the hepatic capsule was evident.

"Livers from mice that did not develop hepatomas were either normal or showed nonspecific parenchymal abnormalities, such as margination of cytoplasm, hyaline, hydropic or fatty changes, and necrosis. Such changes were characteristically patchy and were not localized consistently to any structure in or around the hepatic lobule. No lesions regarded as preneoplastic were observed.

" Other tissues: Degenerative changes were seen in the renal tubules of several animals. Most bladders examined were normal: squamous metaplasia was observed in three mice and epithelial atypia in one. The vesical epithelium in this mouse showed some variation in size and shape of the component cells and their normal regular polarity was somewhat disrupted. Nuclear structures were, however, largely normal, mitotic figures were rarely seen, and there

was no evidence of proliferation into the lumen of the bladder or downward through the basement membrane - the latter structure was intact."

These results clearly fail to implicate any of the known hydroxylated metabolites of 4-aminobiphenyl as the proximate carcinogen derived from 4-aminobiphenyl. Indeed the high ability to produce hepatomas shown by the parent amine is very surprising as it had always been assumed that metabolic activation was a prerequisite for initiation of the carcinogenic response. Similarly, the finding that 4-amino-4'-hydroxybiphenyl was at least as active as 4-amino-3-hydroxybiphenyl, suggests that hitherto unconsidered metabolites may be important under certain conditions.

The activity of the hydroxylated derivatives and 4-aminobiphenyl is difficult to understand at present and it may be that metabolic activation is possible in this test system. If N-hydroxylation is thought to be necessary for carcinogenic activity, then one would have expected the 4-hydroxylamino-biphenyl to be more active than the parent amine. However, it may be that the physical properties of 4-aminobiphenyl allow it to be transported to the liver, converted to the hydroxylamine and, because of the lack of conjugating enzymes, the local concentration of the hydroxylamine in the liver increases. It has been demonstrated that ortho-aminophenol:uridine diphosphoglucuronyl transferase is present in the newborn mouse at about twenty per cent of the level in adults (Dutton & Lawes, 1966).

However, as the hydroxylamine does produce liver tumours, this compound or material derived from it must reach the target organ. Reduction of the hydroxylamine to the amine followed by re-oxidation in the liver, is a

possibility; such a system has been shown to operate in the newborn mouse during the conversion of urethane to N-hydroxyurethane and vice versa (Nery, 1968; Boiato, Mirvish & Berenblum, 1966). This mechanism would also suppose that the other aminophenols are N-oxidised, but at present no real evidence is available to support this hypothesis.

In the light of the results cited earlier (Jondorf et al. 1958; Fouts et al. 1959) there has been a tacit assumption that the newborn animal lacks drug metabolising enzymes; it is now apparent that this is not always the case, and certain processes may proceed at considerable rates. Beckett, Gorrod & Watson (1967) found that liver obtained from human foetuses had high levels of chlorpromazine N-oxidase activity, and Uehleke (1964) observed that the N-hydroxylation of p-chloraniline occurs at approximately the same rate with hepatic microsomes derived from either young or old rats, rabbits or cats. Kato, Takanaka & Onodo (1970) have shown that the young mouse, unlike the young rat, did not have an impaired ability to hydroxylate hexobarbital, demethylate aminopyrine or parahydroxylate aniline. In a study carried out in rats, Basu, Dickerson & Parke (1971) have shown that biphenyl-2-hydroxylase activity is present early in life, but disappears when the animal reaches maturity.

The exact mechanism controlling the depressed levels of certain enzymes in the newborn has not been elucidated. Feuer and Liscio (1969) have implicated the high level of gonadal steroids derived from the mother. If this is so, then the hormonal status of the mothers and/or the reactivity of the test compounds with factors controlling the levels of steroids in the neonate, may well be a factor in producing the results described in this section.

Another striking result of these experiments, is the much greater sensitivity to the compounds of the male animals. Similar results have been obtained in other studies when mice have been exposed to carcinogenic agents during the neonatal period (Klein, 1959; Roe & Walters, 1967). The relative responses of the sexes seen when adult mice are treated with a compound, appears to vary depending upon the amine. 2-Amino-5-azotoluene given to mice from the age of two months produced a higher incidence of hepatomas in females (Andervort, Grady and Edwards, 1942), and in some strains of mice this amine was virtually inactive in males. When this compound was administered to newborn mice it gave rise to hepatomas in males but not in females (Nishizuka, Ito & Nakakuki, 1965).

When a related amide, 2-acetamidofluorene,was given to five different strains of adult mice, no difference in sex susceptibility was apparent (Bonser & Armstrong, 1947). However, Leatham (1951) found that when this compound was given to adult Swiss mice, males were more susceptible than females. This result was also found for Strain A/He mice exposed to 2-acetamidofluorene (Klein, 1959). It is of interest that when the presumed active metabolite of 2-acetamidofluorene, i.e. N-hydroxy-2-acetamidofluorene,was tested in newborn mice by Klein & Weisburger (1966) a high incidence of hepatomas was again found in male mice, whereas this metabolite was inactive in females.

The activity of 2-acetamidofluorene and its N-hydroxylated derivative in producing hepatomas in adult males after treatment of newborn mice, has recently been confirmed by Fujii & Takakashi (1974).

As described in the introduction to this section, 4-aminobiphenyl itself produces higher yields of hepatomas in adult female mice of the C57 X IF strain

(Clayson et al. 1967) than in adult males. These results indicate that a genetic factor is of obvious importance in determining strain susceptibility to carcinogens. The results in this section also indicate that if metabolism of the amino compounds is a prerequisite for carcinogenesis, then a marked sex difference in either extent or nature of metabolic pathways exists. Such sex differences have been shown to exist in rats (Kato, Chiesara & Frontino, 1962) and man (Beckett, Gorrod and Jenner, 1971), but have not previously been observed in mice (Quinn, Axelrod & Brodie, 1958; Novick, Stohler & Swagzdis, 1966).

Davies, Gigon & Gillette (1969) examined the rate of metabolic oxidation of ethylmorphine by male and female rats, and concluded that the difference in oxidation rates was due to differences in the reduction of the ethylmorphine-cytochrome P-450 complex. However, ethylmorphine gives a Type I spectrum on combination with cytochrome P-450 (Remmer et al. 1966), whereas the compounds used in this section all give Type II spectra (Gorrod & Greim, 1968 quoted by Temple, 1971). Gorrod and Greim also showed that the K_s values for 4-aminobiphenyl, 4-amino-4'-hydroxybiphenyl and 4-amino-3-hydroxybiphenyl were all very similar when determined with adult male or female mice cytochrome. No significant difference between the sexes was found in either control or phenobarbitone pretreated mice. When animals were pretreated with 4-aminobiphenyl prior to determining the K_s values, a very marked decrease in K_s values was found with 4-aminobiphenyl and its 4'-hydroxylated derivative. The K_s value of the ortho-aminophenol studied did not change in the male mice so treated and decreased 50% in female mice. As these compounds were equitoxic to male and female mice the results indicate that perhaps the relationship

between K_s and K_m is not as direct as sometimes supposed (Remmer et al. 1966).

The results clearly confirm the newborn mouse as a sensitive test animal for examining the carcinogenic potential of chemical substances. In the above results the carcinogenicity of two substances has been confirmed and the carcinogenic activity of the other two substances has been revealed.

At present, few aromatic amines or their metabolites have been tested by this method. Roe, Mitchley & Walters (1963) examined the bladder carcinogen 2-naphthylamine and its N-hydroxylated derivative in newborn BALB/c mice, and found both produced a slight increase in lung tumours which was a "doubtful but probably positive" result. In a later paper, Walters, Roe, Mitchley & Walsh (1967) re-examined these two compounds at a higher dose level and with injections during the first five days of life. Under these conditions 2-naphthylamine was inactive whilst 2-naphthylhydroxylamine was active in producing pulmonary tumours. No difference was reported in the distribution of these tumours between the sexes.

1-And 2-naphthylamine, 1- and 2-naphthylhydroxylamine and 1- and 2-nitroso-naphthalene were tested by Radomski, Brill, Deichmann & Glass (1971) using newborn Swiss mice. In these tests the parent amines showed only very slight activity, the hydroxylamines produced lung adenomas in female mice and hepatomas in male mice, and 1-nitrosonaphthalene produced lung adenomas in male mice and was inactive in females, whereas 2-nitrosonaphthalene produced hepatomas in male mice but had low activity in the lungs of female mice. The results all indicated that N-oxidation produced more active compounds. In view of the known or suspected carcinogenicity of ring hydroxylated derivatives of the naphthylamines, it is a great pity that the authors did not include any of the ring-hydroxylated compounds in their experiments.

The tumour-inhibitory amine 6-aminochrysene, which is not carcinogenic in adult rats and mice, was highly carcinogenic when given to newborn male mice, both lung and liver tumours being produced (Roe, Carter & Adamthwaite, 1969; Carter & Roe, 1970). It seems that in this test system, aromatic amines and their derivatives which have so far been examined only produce significant neoplasms in lung or liver, and no rule is available to predict which organ will be affected. It is noteworthy that the original interest in these compounds arose from their ability to produce bladder tumours in certain animal species, yet no bladder tumours have been observed following their administration to newborn mice.

It is suggested that this test could be applied to more compounds and their metabolites in the hope that a better understanding of the role of the metabolites in carcinogenesis will evolve.

However, this test system is not without its critics and Toth (1968) has examined all the data on the testing of urethane and N-hydroxyurethane by this method. A fuller survey of the potential of this system is indicated in the review by Porta & Terracini (1969).

If, as discussed in this section, the neonate does have the ability to utilise certain metabolic pathways, then knowledge as to the situation in the human neonate is of obvious importance. Recently, more attention has been paid to this aspect of human drug metabolism. Serini, Morselli & Pardi (1972) have reviewed the situation in man and several routes of metabolism have been detected.

In order fully to exploit the newborn mouse testing technique, it would seem desirable to gain more knowledge of the drug metabolising potential of mice during development. As only very small amounts of test compounds are given to newborn mice this may have to await the development and application of highly sensitive analytical techniques.

SECTION 7 THE INFLUENCE OF AROMATIC AMINES AND CERTAIN OF THEIR HYDROXYLATED DERIVATIVES ON LYSOSOMAL STABILITY

7.1 INTRODUCTION

In 1955 de Duve, Pressman, Gianetto, Wattiaux and Applemans reported on the cellular location of a number of hydrolytic enzymes in rat liver. The enzymes studied were characterised by all having acid pH optima. They could be sedimented by differential centrifugation methods, and all exhibited the property of latency; that is, the enzymes were unable to maximally hydrolyse their substrates until the enzyme preparation had been subjected to certain treatments. The treatments used were: addition of detergents, changes in the osmolarity of the suspending medium, repeated freezing and thawing of the preparation and exposure to ultrasonic waves.

As these treatments were known to have the ability to damage biological lipoprotein membranes, de Duve et al.(1955) suggested that these hydrolytic enzymes were present in the cell sequestered within a membrane. The finding that these latent enzyme preparations had different properties, when examined by ultracentrifugation, than the known cellular organelles, indicated that a new organelle had been recognised. As the original enzymes studied all had lytic properties de Duve et al. called the new cellular particles, lysosomes. The lysosome concept is indicated in Figure 7.1.1., although only the original enzymes studied are indicated. Further studies using both light and electron microscopy have revealed the presence by histochemical techniques of particles within cells, having the properties of lysosomes (Dingle and Fell, 1969; Dingle, 1972). These studies further showed that lysosomes were not an homogeneous population, and that their size and number varied not only from organ to organ but also within different cells of any specific organ. It is now realised that the number of enzymes which can

be described as lysosomal far exceeds the five originally described by de Duve et al.

Tappel (1969) and Barret (1972) showed that at least forty enzyme activities are associated with lysosomes from various tissues, and that they have the potential to degrade virtually every biological macromolecule. In addition, a multiplicity of smaller molecules can also act as substrates for these enzymes.

The finding of lysosomal enzymes within an organelle, yet separated from their substrates by a phospholipid membrane, was something of a paradox. In view of the ability of these enzymes to degrade cellular constituents, it was suggested that the function of lysosomes was to release enzymes upon cell death which could then break down macromolecules, and allow the products to be re-utilised by the organ. It was soon realised that this was really an oversimplification of the situation, as following the administration of many inert compounds such as asbestos, silica or foreign proteins (e.g. horseradish peroxidase or egg albumin), these "foreign" materials appeared within certain lysosomes, particularly those of the kidney or liver tissue (Allison, 1967; Straus, 1967). From this it was concluded that lysosomes probably play some role in phagocytosis, but as this process involves invagination of the cell membrane it would be expected that the lysosomal membrane would have a similar composition to the cell membrane, whereas electron microscopy showed that this was not the case.

Studies on the biosynthesis of the lysosomal enzymes indicated that whilst synthesis occurred on the rough endoplasmic reticulum, complete lysosomes appeared to originate in the region of the Golgi complex. Novikoff, Novikoff, Quintana and Haun (1971) have suggested that lysosomal enzymes are synthesised and transported to smooth endoplasmic reticulum (S.E.R.) adjacent to the Golgi

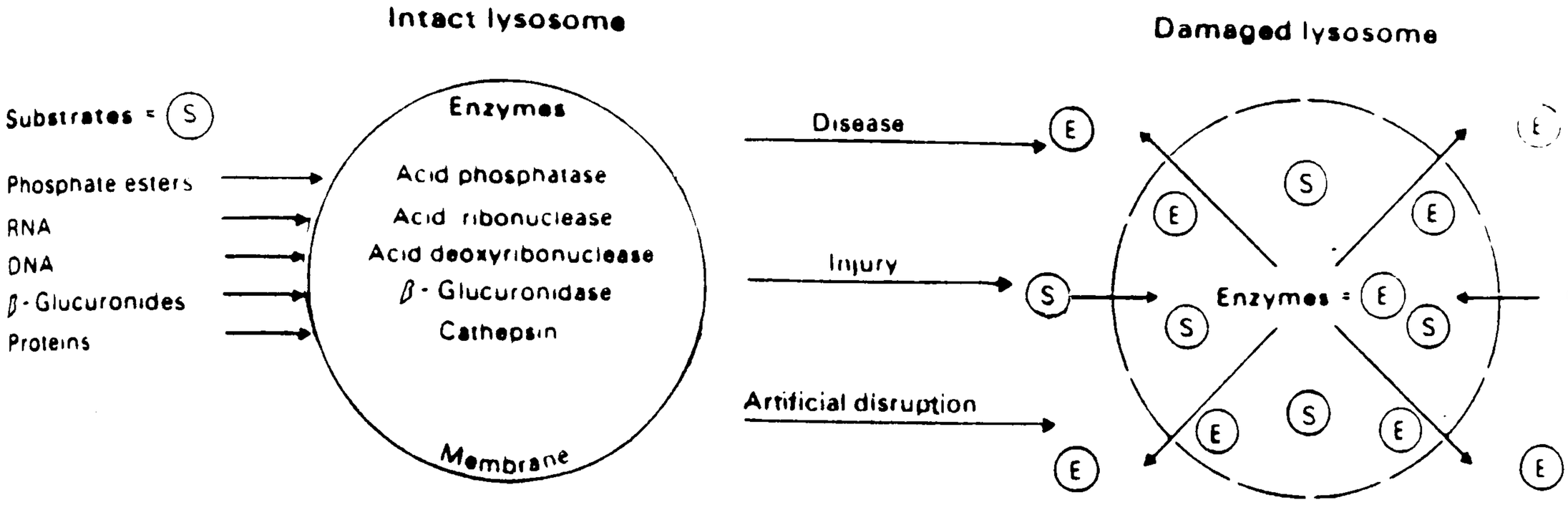


Fig. 7.1.1 Early concept of the role of lysosomes in cellular processes

(after Pitt 1975)

(black areas rich in hydrolytic enzymes)

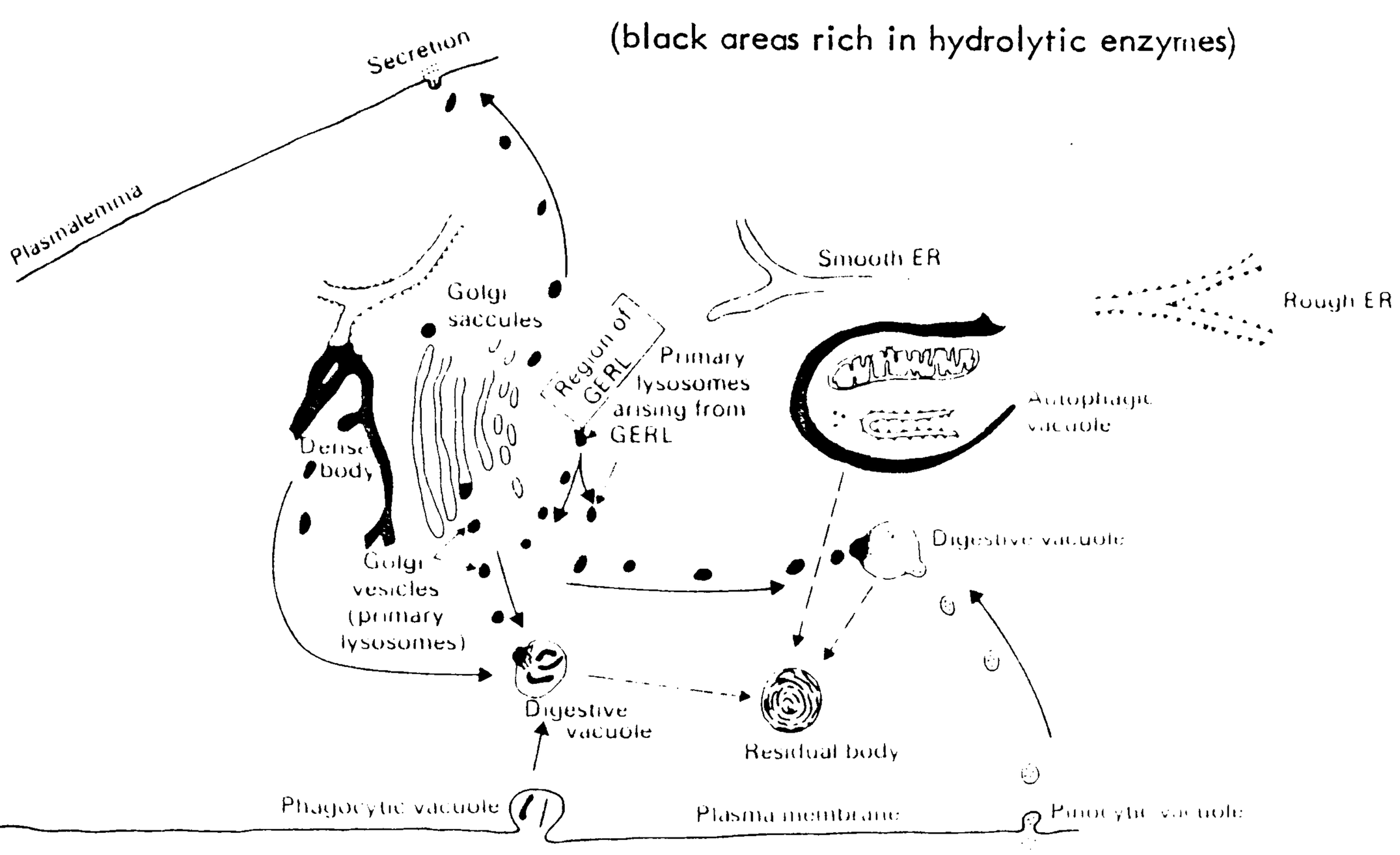


Fig. 7.1.2 Contemporary view of the origins, interrelationships and role of lysosomes

(after Novikoff and Holtzman 1970)

complex; the S.E.R. is then used to encapsulate the enzymes. This system, known as G.E.R.L. (Golgi-endoplasmic reticulum-lysosome), is thought to produce primary lysosomes which can then become involved in a variety of cellular processes. Primary lysosomes have been shown to merge with phagosomes, pinocytic and autophagic vacuoles early after their formation by GERL to form secondary lysosomes. As each of these vacuoles may have a different composition it is easy to understand the heterogeneity of secondary lysosomes, and as secondary lysosomes may fuse with each other, or with another primary lysosome, the situation is extremely complex (Fig.7.1.2).

It is now known that the cellular function of lysosomes is far more intricate than the original "suicide bag" concept, and that lysosomes are intimately connected with such processes as cell metamorphosis and cell differentiation. Lysosomes have been found to occur in all tissues of all animal species examined. More recently it has become obvious that lysosomes are not only involved in intracellular digestion via phagocytosis, pinocytosis and endocytosis, but also in extra-cellular digestion following the release of enzymes from the cell. Apart from their involvement in normal cellular physiology, lysosomes are implicated in a variety of pathological conditions such as diseases of connective tissue and rheumatoid arthritis, as well as a variety of "storage" diseases. The details of these many facets of lysosome physiology and pathology are too numerous to elaborate herein, and excellent monographs are available by Dingle & Fell (1969) and Dingle (1973).

Allison (1967, 1969) has proposed that lysosomes may be involved in the carcinogenic process. The evidence in support of this concept is detailed by Allison (1969).

Allison's theory requires that carcinogenic substances are localised within the cellular lysosomes of susceptible tissue, and thereby cause sufficient damage to the lysosomal membrane to allow the release of desoxyribonuclease (DNA-ase). It is suggested that DNA-ase has the ability to cross the nuclear membrane and hydrolyse desoxyribonucleic acid and cause chromosomal damage.

In support of this theory, Allison & Mallucci (1964) demonstrated that carcinogenic hydrocarbons were taken up by four different tissues in tissue culture, and that the hydrocarbons were localised within the lysosomes. In these experiments the non-carcinogenic hydrocarbon, anthracene, had the same tissue distribution as the carcinogenic compounds studied. This suggested that localisation per se was not enough to account for the difference in specificity of activity exhibited by different hydrocarbons, and that perhaps better correlation could be obtained if the metabolism of the compounds was considered.

In a study of rat adrenal necrosis produced by 7,12-dimethylbenzanthracene (DMBA), Allison and Dingle (1966) showed that one metabolite, viz. 7-hydroxymethyl-12-methylbenzanthracene (7-OHM-12-MBA), had the ability to release acid phosphatase from adrenal lysosomes, the isomeric metabolite 12-hydroxymethyl-7-methylbenzanthracene (12-OHM-7-MBA) being far less active. The activity of 7-OHM-12-MBA was much greater towards adrenal tissue than towards either kidney or liver tissue preparations. As adrenal necrosis can also be produced in vivo by administration of 7-OHM-12-MBA to rats, (Boyland, Sims and Huggins, 1965) but not by the isomeric compound, clearly a tissue-

specific, metabolite-specific cellular interaction had been demonstrated.

In another study on the role of lysosomes in carcinogenesis, Allison and Paton (1965) showed that human diploid cells had the ability to concentrate a variety of photosensitizing agents within lysosomes. When these preparations were further treated with light, of a suitable wavelength, chromosomal damage occurred. This damage to the chromosomes was not seen in preparations not irradiated, or not treated with the photosensitizing agent but irradiated. The authors conclude that it was the combined action of the localisation of the material within lysosomes, plus the photosensitizing effect of light, producing damage to the lysosomal membrane and thus allowing leakage of DNA-ase into the cytoplasm. It has previously been shown that addition of DNA-ase to isolated nuclei caused the DNA-dependent RNA polymerase to stop (Widnell and Tata, 1964), indicating that DNA-ase can traverse the nuclear membrane. It is interesting that lysosomal DNA-ase has different properties from pancreatic DNA-ase; the former, being double stranded, seems to be able to cleave both strands of the DNA simultaneously, whilst the latter breaks only one strand of the DNA helix. It is suggested that the double strand break may be an irreparable process leading to excision of genetic material and ultimately to the neoplastic condition (Allison, 1967, 1969).

Despite the evidence presented above, knowledge regarding the involvement of lysosomes in carcinogenesis is still incomplete. For example, it is known that lysosomes are labilised as a result of exposure to a variety of toxic substances, and yet the tissue never develops into the neoplastic state. If the toxicant is indeed so toxic that cell death occurs, then it is obvious that carcinogenesis cannot proceed. However, Kerr (1973) has reviewed numerous changes which

occur in the lysosomes of liver cells as a result of sub-lethal injury where development to frank tumour could have occurred. It may be that in these cases either the toxicant has the additional ability to inhibit the activity of the lysosomal enzyme(s), so that only functionally inactive enzyme(s) is released from the organelle, or that a stabilising factor is present which selectively controls which enzymes are released. Many materials occurring in nature, particularly certain steroids, do have the ability to "protect" or stabilise lysosomal membranes under conditions where damage is normally expected (Weissmann, 1969).

Despite the objections stated above, further evidence does indicate that lysosomal changes occur during the change from normal to cancer tissue. Maisin, Maldgune and Deckers-Passau (1961) found an increase in cathepsin, acid DNA-ase and acid ribonuclease in the livers of rats fed diets containing either 2-acetamidofluorene or 4-dimethylaminoazobenzene. After feeding 2-acetamidofluorene to rats at low but carcinogenic levels, Flaks (1970) found an increase in the number of lysosomes present in the liver cells at 3-4 weeks and 8-12 weeks of feeding. These lysosomes were present associated with autophagic vacuoles and dense residual bodies. On prolonged feeding of 2-acetamidofluorene for 8-10 months the number of lysosomes and autophagic vacuoles decreased compared with the number present earlier; however the abundance of these organelles was still sufficiently greater than normal to be well recognised (Flaks, 1971).

The publication by Flaks (1970, 1971) using electron microscopy techniques extends and confirms the earlier work of Mikata and Luse (1964).

In addition many other hepatocarcinogens have been reported to increase the levels of lysosomal enzymes. Amongst the compounds studied are aflatoxin (Pokrovsky, Kravchenko & Tutelyan, 1972) and dimethylnitrosamine (Slater & Greenbaum, 1965).

From the foregoing it seemed desirable to investigate the possible role of lysosomal damage in carcinogenesis induced by aromatic amines. In order to achieve meaningful results it is obvious that the target organ from a species susceptible to amine carcinogenesis has to be used as a source of lysosomes. This is necessary as lysosomes vary both in enzyme content and membrane fragility depending upon both organ and species.

If lysosomal damage is involved in amine carcinogenesis, then the damage should be caused by the metabolite of the amine responsible for initiating the carcinogenic process, and not by the parent amine or the non-carcinogenic hydroxylated metabolites. A similar approach has previously been used by Gorrod, Alifano, Papa and Quagliariello (1967) in a study of mitochondrial damage produced by aromatic amines and some hydroxylated derivatives. These authors showed that whilst aromatic amines were without effect on mitochondrial architecture, certain aminonaphthols produced an immediate gross lytic effect, with concomitant inhibition of respiration and phosphorylation.

7.2 EXPERIMENTAL

7.2.1 Materials

Aniline, ortho-aminophenol, para-aminophenol, anthranilic acid and 1- and 2-naphthylamines were purchased from commercial sources. Hydroxylated derivatives of 1- and 2-naphthylamines were a gift from Dr.D.Manson. Phenylhydroxylamine and ortho-carboxyphenylhydroxylamine were a gift from Dr.R.Nery. 4-Aminobiphenyl and its hydroxylated metabolites were prepared as described in Section 2. 3-Hydroxyanthranilic acid was a gift from Dr.P.Grover and 5-hydroxyanthranilic acid was synthesised according to published methods. All substances were purified by recrystallisation until chromatographic examination indicated the substance was pure. All melting points agreed with the literature values. Triton X-100 was a gift from Rhom & Hass Ltd. Tween 20, Span 20 and Brij 35 were supplied by Honeywell-Atlas Ltd; Cetrimide by I.C.I.Pharmaceuticals Ltd.; and sodium lauryl sulphate by B.D.H.Ltd.

7.2.2. Tissue Preparations

Urinary bladders of dogs were obtained as soon after death as possible and rinsed with isotonic sucrose solution. The bladders were kept in ice-cold isotonic sucrose until use, which was always within an hour of the animal's death. The dogs were usually greyhound or alsatian strains, being primarily used as donors for organ transplant experiments. The donor animal was sacrificed by Nembutal overdose just prior to the excision of the urinary bladder.

Histochemical examination of dog bladder tissue by Dr.E.Katchburian of the Department of Histopathology, The Royal Post-Graduate Medical School, revealed the presence of various lysosomal enzymes only in the epithelial cells (Gorrod, 1967).

The epithelial cells were removed from the muscle wall of the urinary bladder by gently scraping the exterior surface of the bladder with a scalpel, the bladder being stretched on a dissection board during this procedure. The epithelial cells were harvested by suspending the scrapings in 0.25 M sucrose/tris 0.05M and centrifuging the suspension for 20m at 1000 xg.

Homogenisation of the epithelial cells was carried out in isotonic sucrose media using a Teflon-glass Potter-Elvejhem homogeniser (Arthur H.Thomas) size B. The glass mortar was held in a specially constructed perspex holder contained in an ice-bath. The teflon pestle was driven by an electric motor at 980 r.p.m., and passed through the cell suspension at least three times with pauses of at least 20 s between passes to ensure adequate cooling.

The homogenate was centrifuged at 600 xg for 5 m. to sediment whole cells, cell debris and nuclei. The lysosome-rich fraction was sedimented at 10,000xg for 20 m. the microsomal fraction was sedimented at 140,000xg for 60 m. and the soluble fraction was that remaining in the supernatant. This technique was essentially as described by Dingle (1961) for the preparation of lysosomes from liver tissue.

The lysosomal sediment was carefully resuspended in 0.25M sucrose/tris 0.05M (pH 7.4), filtered using nylon sifting cloth grade 9N (Henry Simon Ltd. Stockport), and diluted with sucrose/tris (pH 7.4) so that the final suspension gave a 40% transmission reading when examined using an EEL nephelometer. This method of standardisation of biological particles in suspension has previously been used by Hübscher (1966) in studies on the epithelial cells of rabbit intestinal tract. This lysosomal suspension was used for the experiments on the properties of dog

bladder epithelial cell lysosomes, and the effects of aromatic amines and some of their hydroxylated derivatives on lysosomal stability.

7.2.3 Enzyme and Protein Assays

7.2.3.1 β-Glucuronidase

β-Glucuronidase activity was determined by measuring the amount of phenolphthalein released from phenolphthalein-mono-β-glucosiduronic acid (Koch-Light Laboratories Ltd) at pH 4.6 and 37° as described by Talalay, Fishman and Huggins (1946). The reaction was stopped and the colour developed by the addition of glycine/NaOH buffer (0.3M, pH 10.6). The absorption was measured at 552 nm. One unit is that amount of enzyme which liberates 1μg of phenolphthalein per hour under the assay conditions.

7.2.3.2 Acid phosphatase

The activity of acid phosphatase was determined by the method of Torriani (1960) using para-nitrophenylphosphate (B.D.H. Ltd.) as substrate at pH 5.0. The para-nitrophenol liberated was measured at 410 nm after the addition of glycine/NaOH buffer (0.3M, pH 10.6) to stop the reaction. A unit of acid phosphatase is that amount of enzyme which liberates 1 μg of para-nitrophenol per hour under the assay conditions.

7.2.3.3 Aryl sulphatase

Aryl sulphatase activity was measured using the method developed by Roy (1953) and modified by Boyland, Wallace and Williams (1955) using para-nitrocatechol sulphate, synthesised according to Roy (1953), as substrate at pH 5.6. The para-nitrocatechol released was measured at 520 nm in the presence of alkaline hydroquinone. One unit of aryl sulphatase activity is that amount of enzyme which liberates 1μg of para-nitrocatechol per hour under the assay conditions.

7.2.3.4. Protease (Cathepsin)

Cathepsin activity was determined using the method of Anson (1939) utilising bovine haemoglobin (Armour Pharmaceutical Co.), purified as described by Gianetto and de Duve (1955), as substrate at pH 3.6. After the protein had been precipitated with trichloracetic acid (6.7%) and removed by filtration, the liberated tyrosine was determined, on an aliquot of the filtrate, by the method of Miller (1959).

A unit of cathepsin activity is defined as that amount of enzyme which liberates 1 μg of tyrosine per hour under the assay conditions.

7.2.3.5. Protein determination

Proteins were determined by the method of Lowry, Roseborough, Farr and Randall (1951), using the modification of Miller (1959). Folin-Ciocalteau reagent (B.D.H. Ltd.) was used and the colour, measured at 730 nm was compared with those of standard solutions of bovine serum albumin (B.D.H. Ltd) subjected to the assay procedure.

7.2.4 Properties of lysosomal preparations from dog urinary bladder epithelial cells

7.2.4.1 The distribution of lysosomal enzyme activity in cell fractions derived from dog urinary bladder epithelial cells

The specific activities of β-glucuronidase, arylsulphatase, acid phosphatase and cathepsin were determined using the assay procedures described in 7.2.3 on various cell fractions prepared as described in 7.2.2. All enzyme activities were determined in the presence of 0.1% Triton X-100 in order to release the enzymes from organelles (Wattiaux and de Duve, 1956).

7.2.4.2 The pH optima of lysosomal enzymes derived from lysomes prepared from dog urinary bladder epithelial cells

A lysosomal preparation, prepared as described in 7.2.2, was treated with Triton X-100 at a final concentration of 0.1%, and stored for one hour at 0°. The suspension was centrifuged at 25,000xg for 10m and the supernatant was assayed for β-glucuronidase, acid phosphatase, aryl sulphatase and cathepsin activities at various hydrogen ion concentrations between pH 2 and 7.

7.2.4.3 The effect of detergents on the release of enzymes and protein from a lysosomal preparation

Aliquots of a lysosomal preparation, prepared as described in 7.2.2, were treated with either sodium lauryl sulphate, Tween-20, Span-20, Triton X-100, Brij 35, or Cetrimide at a final concentration of 0.1%. The aliquots were incubated at 37° for 10m and then centrifuged at 25,000xg for 10m. Enzyme and protein assays were then carried out on the supernatant as described in 7.2.3.

7.2.4.4 The effect of temperature on the release of enzymes from a lysosomal preparation

Aliquots of a lysosomal preparation in 0.25M sucrose/tris (pH 7.4) were stored at either -10, 0, 20 or 37° for 1 h, after which they were centrifuged at 25,000xg and the supernatant was assayed for protein and β-glucuronidase, aryl sulphatase, acid phosphatase and cathepsin activities. A further aliquot was centrifuged at zero time and another was treated with Triton X-100 for 1 h at 37° prior to centrifuging, and the supernatant assayed as above.

7.2.4.5 The effect of incubation time on the release of enzymes from a lysosomal preparation

A lysosomal preparation, prepared as in 7.2.2, was incubated at 37°. Aliquots

were removed at various time intervals and centrifuged at 25,000xg for 10m. The supernatant was assayed for β-glucuronidase, aryl sulphatase, acid phosphatase and cathepsin activity and protein as described in 7.2.3. The total activities were determined after treatment with Triton X-100, 0.1%, and centrifugation at 25,000xg for 10m.

7.2.4.6 The effect of pH on the release of enzymes from a lysosomal preparation

A lysosomal preparation was recentrifuged at 10,000xg for 20 m and the sediment resuspended in 0.5M sucrose. Aliquots of this suspension were then diluted with equal volumes of either acetate buffer (0.1M), pH 4.6, 5.6 or 6.6,or tris buffer (0.1M) pH 7.6 or 8.6, and incubated at 37° for 1 h. The aliquots were then centrifuged at 25,000xg for 10 m and the enzyme and protein level in the supernatant determined. The total activity was determined in the presence of Triton X-100, 0.1%.

7.2.4.7 The effect of repeated freezing and thawing on the release of enzymes from a lysomal preparation

A lysosomal preparation from dog urinary bladder epithelial cells was divided into equal portions, and one aliquot immediately centrifuged at 25,00xg for 10 m, and protein and enzyme assays carried out on the supernatant as described in 7.2.3. The remaining aliquots were frozen in liquid nitrogen for 2 m, after which they were allowed to thaw by placing in an ice-water bath at 0-4°. One aliquot was centrifuged as before, and the supernatant assayed as above. Freezing in liquid nitrogen for 2 m and subsequent thawing was repeated on the remaining fractions, one aliquot being removed, centrifuged and the supernatant assayed at each successive thawing. The supernatant obtained in each case was analysed for

protein and enzyme activity. The total enzyme activity was determined in the presence of Triton X-100, 0.1%. Each aliquot took approximately 5m to freeze and thaw.

7.2.4.8 The effect of certain solvents on the release of enzymes from a lysosomal preparation

A lysosomal preparation was separately treated with absolute ethanol, acetone or dimethylsulphoxide at a final concentration of 1%, and incubated at 37° for 1 h. Duplicate aliquots were treated with the solvent plus Triton X-100, 0.1%. One aliquot was centrifuged at 25,000xg for 10m at zero time, the remainder were centrifuged as above after incubation for 1 h. The supernatants were assayed for enzyme activity using the methods described in 7.2.3.

7.2.4.9 The determination of lysosomal fragility

A lysosomal preparation, prepared as described in 7.2.2, was allowed to reach 37°, and an aliquot immediately removed and centrifuged at 25,000xg for 10m. Enzyme and protein assays were carried out on this supernatant as described in 7.2.3. The lysosomal preparation was maintained at 37° for 1 h and another aliquot removed and centrifuged at 25,000xg for 10 m. Enzyme and protein assays were carried out on this supernatant. The total enzyme activity of the preparation was determined in the presence of Triton X-100 at a final concentration of 0.1% as described by Wattiaux and de Duve (1956).

The "bound" activity is defined as the "total" activity minus the activity found in the supernatant at zero time. The lysosomal fragility can then be defined as that percentage of the "bound" activity which becomes non-sedimentable, under the centrifugation conditions used, after treatment at 37° for 1 h (Dingle, 1972).

7.2.5 The effect of aromatic amines and some of their hydroxylated derivatives on the release of enzyme activity from lysosomes prepared from dog urinary bladder epithelial cells

Lysosomal preparations, prepared as in 7.2.2, were treated with aromatic amines and certain of their hydroxylated derivatives at a final concentration of 0.5mM (unless otherwise stated). The compounds were added as solutions in dimethylsulphoxide, such that the final concentration of the solvent was 1%. Control experiments in which the test compounds were omitted, but the solvent included, were always run parallel to the test system. Duplicate experiments were carried out in which Triton X-100, 0.1%, was included in the test system as well as the test compounds plus solvent.

Aliquots of the lysosomal preparation obtained at zero time, and an aliquot treated with Triton X-100, 0.1%, were centrifuged at 25,000xg for 10m and the supernatants were assayed for enzyme activity as described in 7.2.3. The test system, with and without Triton X-100, 0.1%, was incubated at 37^{o} for 1 h, then centrifuged at 25,000xg for 10m and the supernatants assayed for enzyme activity.

In some experiments the concentration of the test compound was varied, the concentration of dimethylsulphoxide being kept constant at 1%.

7.3 RESULTS AND DISCUSSION

The experiments described in the previous section (7.2.4) were carried out to examine the possibility of obtaining a cell fraction from dog urinary bladder epithelial cells, which had the property of latency in respect to certain hydrolytic enzymes. These enzymes are known to be associated with lysosomes derived from other organs. The subcellular distribution of enzymes and protein is shown in Table 7.3.1. The results tabulated are from one specific tissue preparation derived from four separate dog urinary bladders. These results show that four hydrolytic enzymes, commonly used as markers for lysosomes in tissue distribution studies, are present in preparations from dog urinary bladder epithelial cells. The table also shows that the highest specific activity of the enzymes studied is always associated with the lysosomal fraction. However, the distribution of the enzymes shows wide variations, and in the case of both aryl sulphatase and acid phosphatase the bulk of the enzyme activity is present in the supernatant soluble fraction. Whether this is a true reflection of the in vivo situation, or whether produced by the homogenisation technique used, is not at present known. In other experiments using differing times of homogenisation, or using the Ultra-Turrax homogeniser, similar high levels of acid phosphatase and aryl sulphatase have been found in the supernatant fraction. This contrasts with the in vivo situation where Gorrod (1967) and Birkbeck and Grover (1965) reported the localisation of acid phosphatase within lysosomes in this tissue.

As observed by others (see Dingle, 1973) considerable variation in enzyme localisation occurred in different preparations, even when the homogenisation technique was strictly controlled. Whilst the results presented in Table 7.3.1

Table 7.3.1 Distribution of protein and enzyme activities in post- nuclear sub- cellular fractions prepared from dog urinary bladder epithelial cells.

Fraction[x]	Protein		Cathepsin			Aryl Sulphatase			β-Glucuronidase			Acid Phosphatase		
	mg	%	Units[+]	%	Sp.Ac.[o]	Units[+]	%	Sp.Ac.[o]	Units[+]	%	Sp.Ac.[o]	Units[+]	%	Sp.Ac[o]
Lysosomal	17	12	3750	41	223	576	14	34	135	49	8.0	104	18	6.2
Microsomal	20	15	1530	17	75	240	6	12	65	24	3.2	47	8	2.3
Soluble	102	73	3840	42	38	3210	80	32	75	27	0.7	418	74	4.1

x Fractions prepared as described in 7.2.2

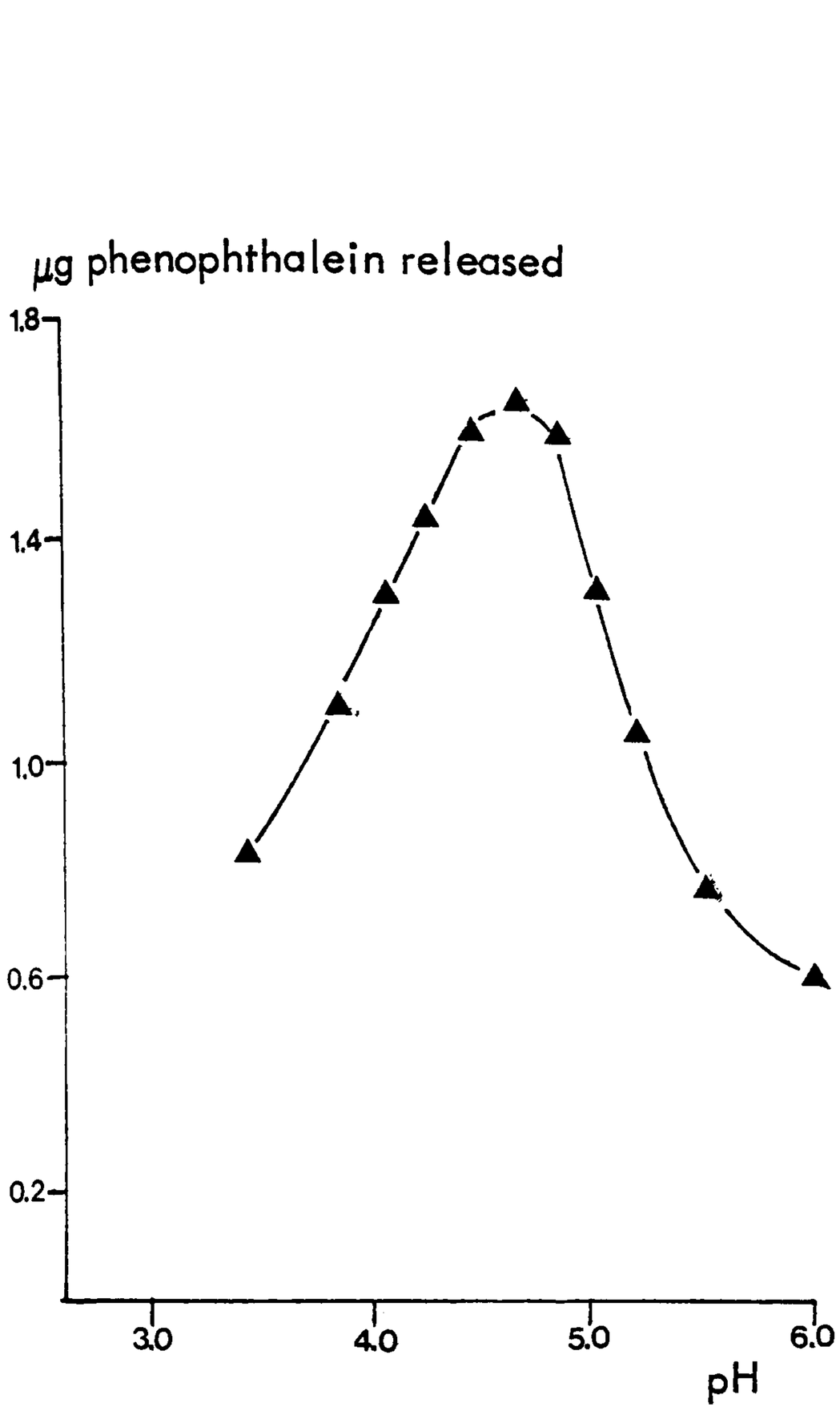
\+ Enzyme units as defined in 7.2.3

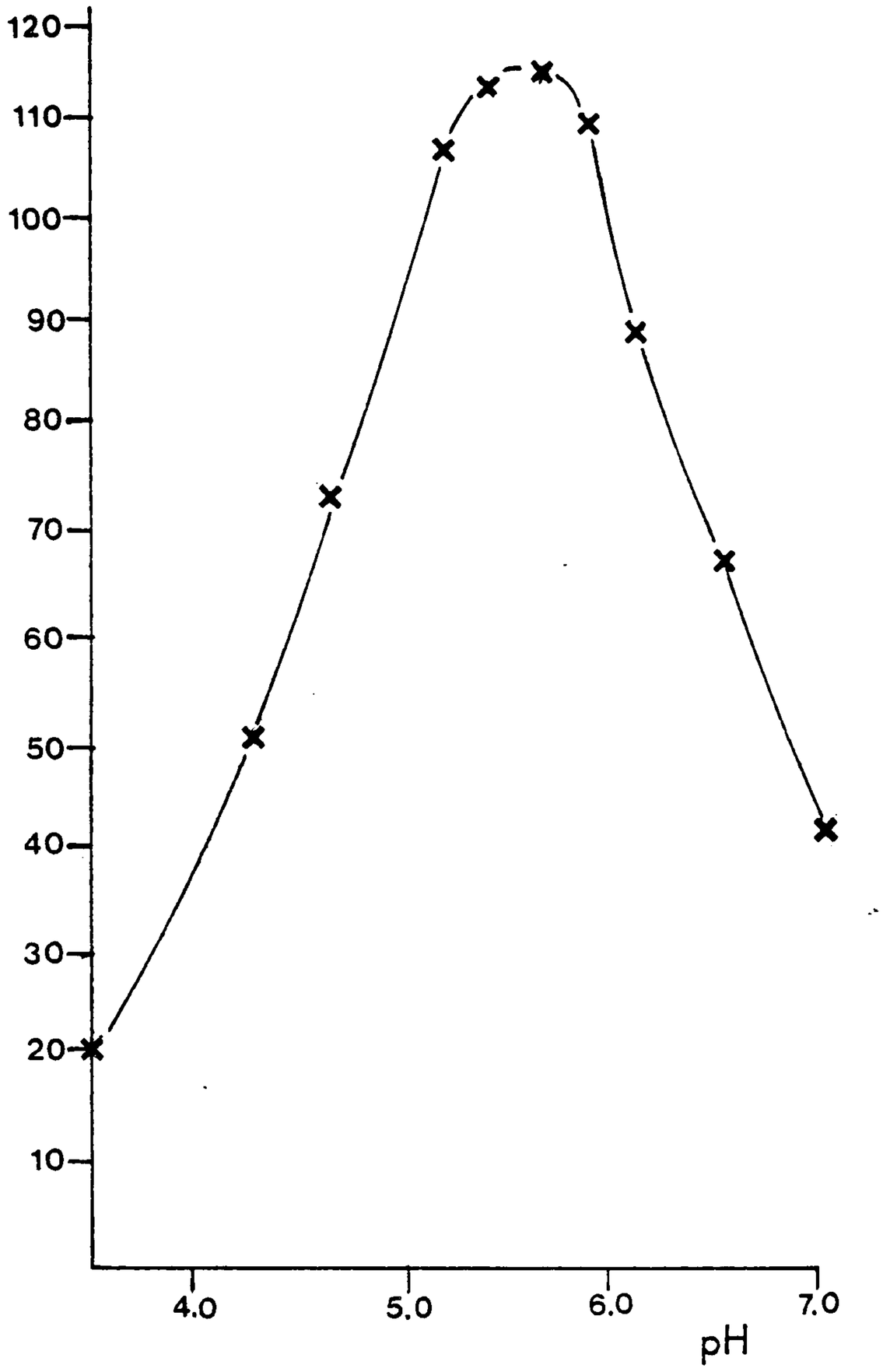
o Sp.Ac. is the activity of the enzyme per mg of protein.

are typical, the percentage of enzyme activity present in the lysosomal fraction varied from 20-50% for β-glucuronidase, 30-50% for cathepsin, and 10-20% for aryl sulphatase and acid phosphatase. There was also a lack of consistency in the ratios of the enzyme activities in various preparations. However, this variation appears to be common in studies of this type, even with easily homogenised tissue such as liver. "Tough" tissue such as muscle, skin, or connective tissue invariably showed a greater variation.

It has previously been reported that lysosomal enzymes may differ in regard to their pH optima compared to the non-lysosomal enzyme (Mills, Paul & Smith, 1953; Ganschow & Paigen, 1967; de Duve, Pressmann, Gianetto, Wattiaux & Appelmans, 1955). It was of interest to check the pH optima of the enzymes found associated with the lysosomal fraction in the dog bladder epithelial preparation. The pH activity curves are shown in Fig.7.3.1; in each case the optimum pH of enzyme activity was in the acid region, a property of many lysosomal enzymes. The pH optimum for each enzyme corresponded to that of the lysosomal enzymes derived from other mammalian tissues.

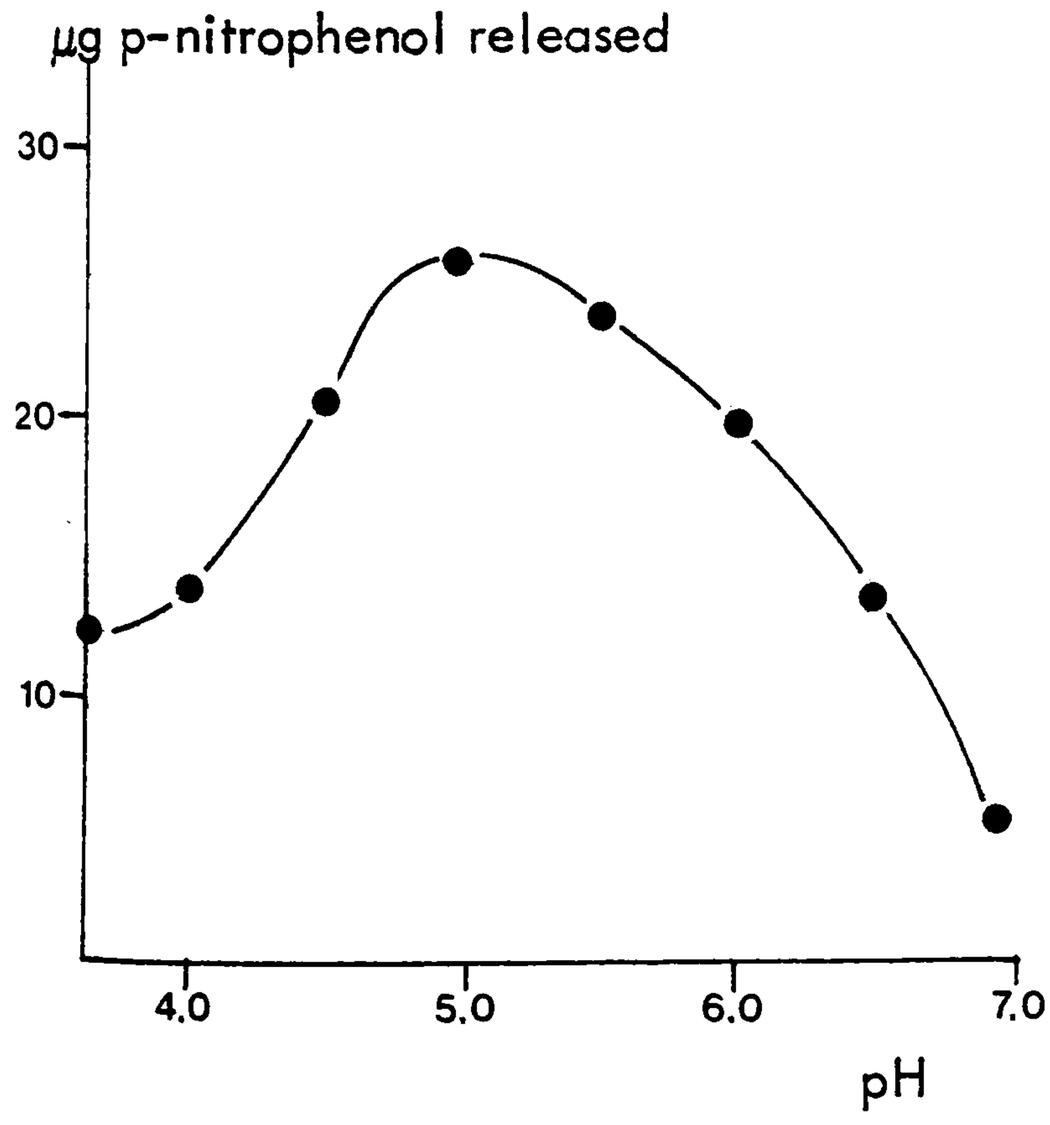
In order to see whether the fraction sedimenting at 10,000xg could really be termed a lysosomal fraction, it was necessary to confirm that it had the property of latency, that is, that certain treatments were able to enhance the level of enzyme activity. In his classic studies, de Duve found that treatment with detergents was able to liberate certain enzymes from a bound form. The results in Table 7.3.2 show that of the detergents studied Triton X-100 had the greatest ability to convert β-glucuronidase and acid phosphatase into a non-sedimentable form. The results also show that both sodium lauryl sulphate and

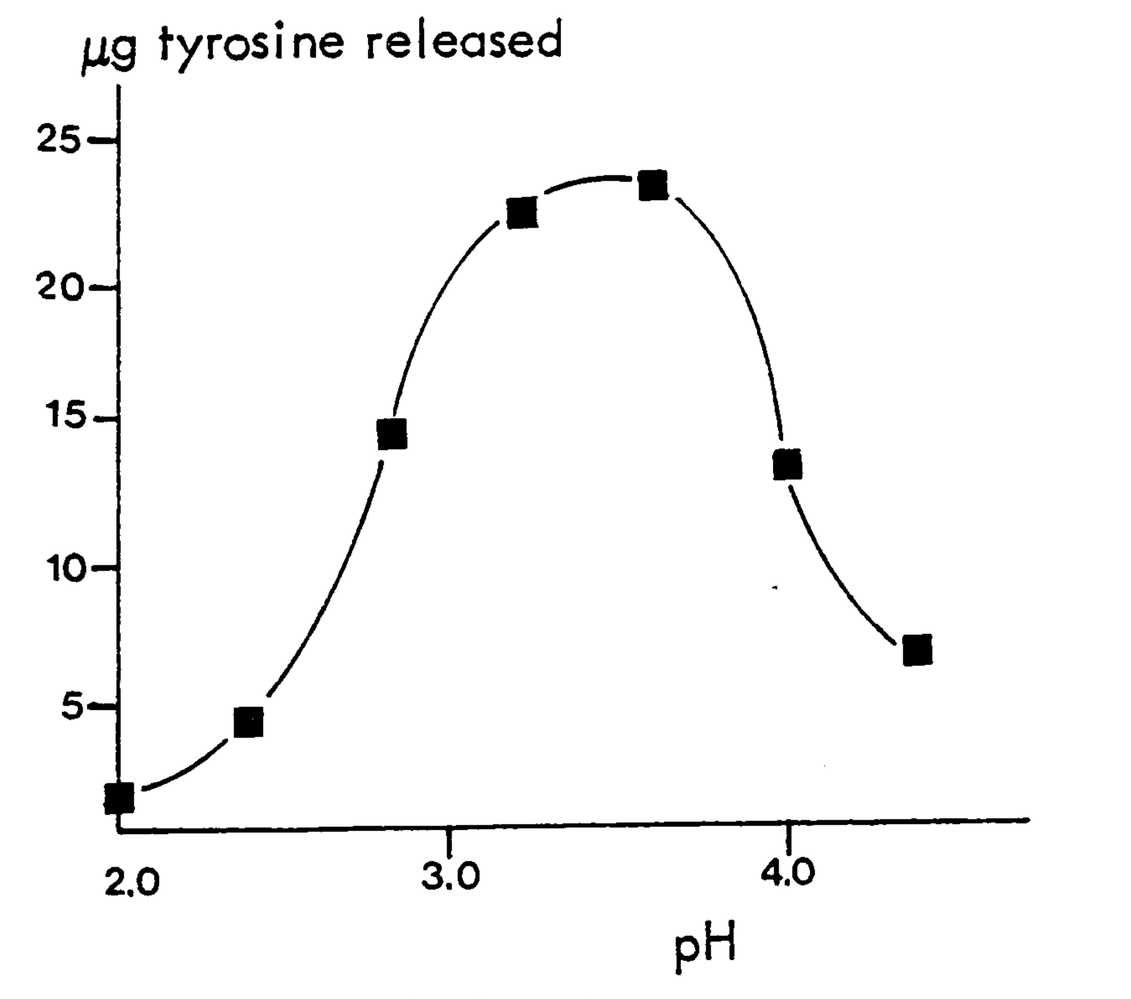




β-Glucuronidase

Aryl Sulphatase





Acid Phosphatase

Cathepsin

Fig.7.3.1. pH activity curves for lysosomal enzymes derived from dog urinary bladder preparations

Cetrimide caused a marked inhibition of the β-glucuronidase activity. Lauryl sulphate, which also inhibited acid phosphatase, had the greatest ability to convert the protein present in the preparation into a form non-sedimentable under the standard centrifugation conditions used. These results are in line with those obtained with preparations from other tissues (Dingle, 1973), and confirm the usefulness of Triton X-100 in releasing enzymes from cellular organelles (Wattiaux & de Duve, 1956; Glassman, 1950; Walker & Levvy 1951, 1953), without causing enzyme inhibition. Triton X-100, a non-ionic detergent, was used in all further experiments to release enzymic activities from "bound" preparations.

A further parameter often used to demonstrate enzyme latency is the variation in the release of the enzymes when the organelle is exposed to various temperatures. The results from one such experiment are tabulated in Table 7.3.3. Incubation of the organelle preparation at 0° caused minimal release of the bound enzymes; at this temperature β-glucuronidase, aryl sulphatase and cathepsin were all released to about the same extent, whereas acid phosphatase was much more labile. Decreasing or increasing the temperature of incubation increased the percentage of bound enzyme released in every case. Again, differences in sensitivity to temperature changes were seen with different enzymes. Increasing the incubation temperature from 0° to 37° trebled the activity of the aryl sulphatase released, the β-glucuronidase being increased five-fold. Acid phosphatase release was increased only from 18 to 25% of the bound material.

Table 7.3.2 The effect of detergents on the release of some enzymes and protein from a dog urinary bladder preparation

Detergent[+]	β-Glucuronidase Units/ml	Acid Phosphatase Units/ml	Protein µg/ml
Nil	1.0	12.8	40
Sodium Lauryl Sulphate	0.2	2.3	132
Tween 20	1.2	10.6	63
Span 20	2.0	26.0	68
Triton X-100	7.5	51.8	83
Brij 35	2.4	18.6	59
Cetrimide	0.4	20.6	50

[+] at a final concentration of 0.1%.

Table 7.3.3 The effect of incubation temperature on the release of some enzymes and protein from a dog urinary bladder preparation

Incubation temp. degrees	% of Bound Material released during 1 h incubation: Protein	β-Glucuronidase	Aryl Sulphatase	Cathepsin	Acid Phosphatase
-10	20	22	20	10	24
0	10	5	7	6	18
20	15	15	15	8	20
37	22	27	21	11	25

Table 7.3.4. The effect of incubation time on the release of β-glucuronidase and acid phosphatase from a dog urinary bladder preparation

Time (m)	Percentage of Bound Enzyme Released+	
	β-glucuronidase	Acid phosphatase
20	3	21
40	8	29
60	13	34
90	18	41
120	24	48
150	29	53

+ at 37° pH 7.4.

Table 7.3.5 The effect of pH on the release of enzymes from a dog urinary bladder preparation

	Percentage of Bound Enzyme Released during 1 hr incubation at 37°			
pH	β-Glucuronidase	Aryl Sulphatase	Acid Phosphatase	Cathepsin
4.6	49	81	21	11
5.6	31	26	18	4
6.6	22	21	21	4
7.6	12	21	25	11
8.6	19	31	32	24

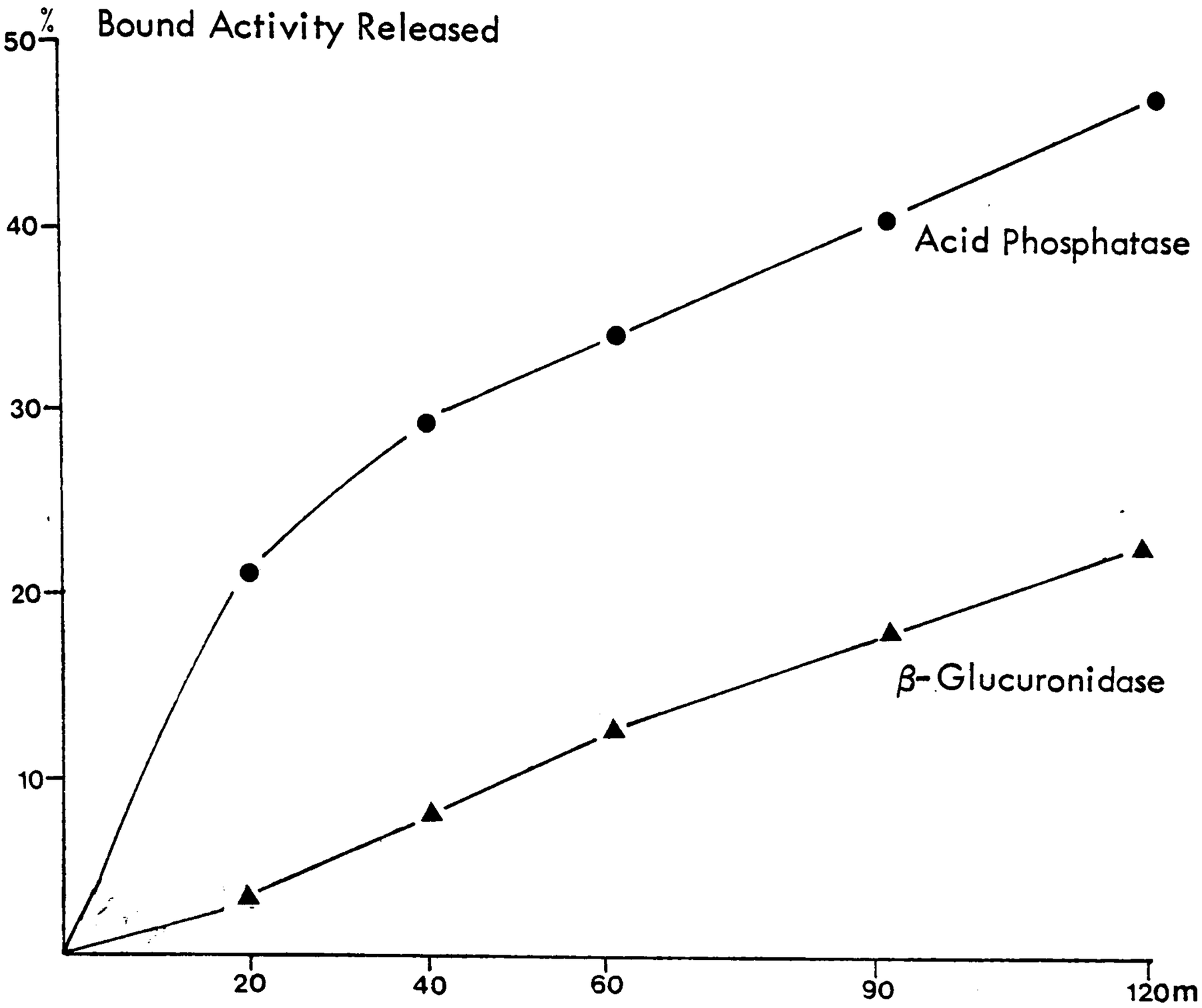


Fig.7.3.2. The influence of incubation time on the release of acid phosphatase and β-glucuronidase from a dog bladder epithelial cell preparation

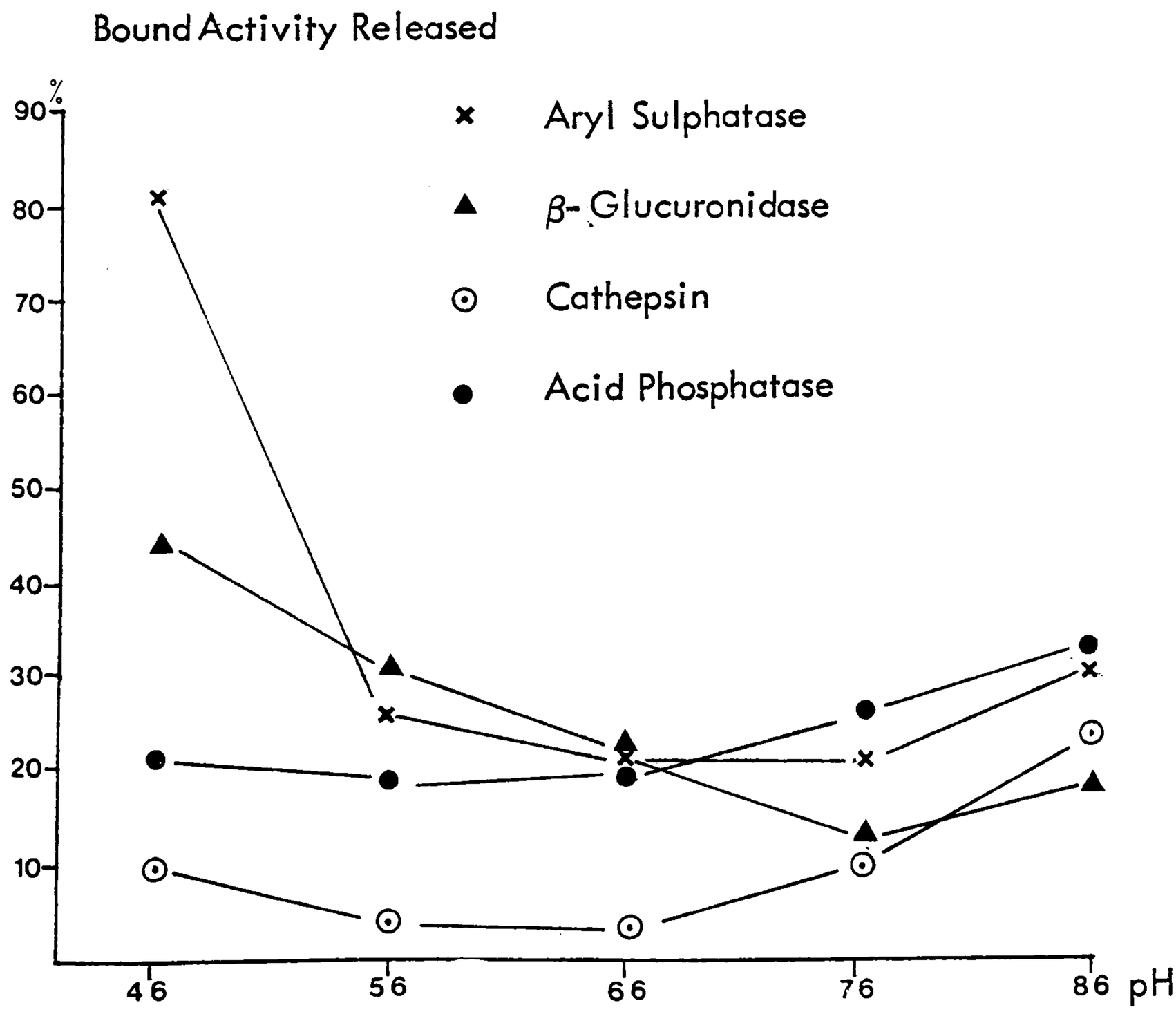


Fig.7.3.3. The influence of pH on the release of bound lysosomal enzymes from dog bladder epithelial cells

When the incubation temperature was kept constant at 37°, and the liberated β-glucuronidase or acid phosphatase was measured at different times, again a difference in sensitivity was observed (Table 7.3.4, Fig.7.3.2). Whilst the β-glucuronidase appeared to be released at a linear rate over the 150 m of incubation, the acid phosphatase was rapidly released during the early period followed by a slow linear release as the incubation time increased.

This variation in sensitivity was also seen when the tissue preparation was exposed to different hydrogen ion concentrations during the incubation. Whilst pH 7.6 seemed to favour the retention of β-glucuronidase within the organelle, a more acid environment (pH 5.6) provided more stability for acid phosphatase and cathepsin. Reducing the pH to 4.6 allowed the release of over eighty percent of the aryl sulphatase activity and about half of the β-glucuronidase activity; in contrast only eleven percent of bound cathepsin was released (Table 7.3.5, Fig.7.3.3).

Following repeated freezing in liquid nitrogen and subsequent thawing at 0°, a gradual release of enzymes and protein was found. These results are shown in Table 7.3.6,and Fig. 7.3.4 confirms the results obtained earlier which indicate that several hydrolytic enzymes are present in dog urinary bladder epithelial cells.

The finding that changes in the external environment of a subcellular fraction, by either incorporation of detergents, changes in pH or changes in temperature or freezing and thawing, allow the release of enzyme and protein, all point to the conclusion that these enzymes are sequestered within a membrane .

The properties exhibited by the subcellular fraction indicate that it is similar in many respects to fractions prepared in a similar manner from other organs and known to contain lysosomes. From the foregoing it must be concluded that a

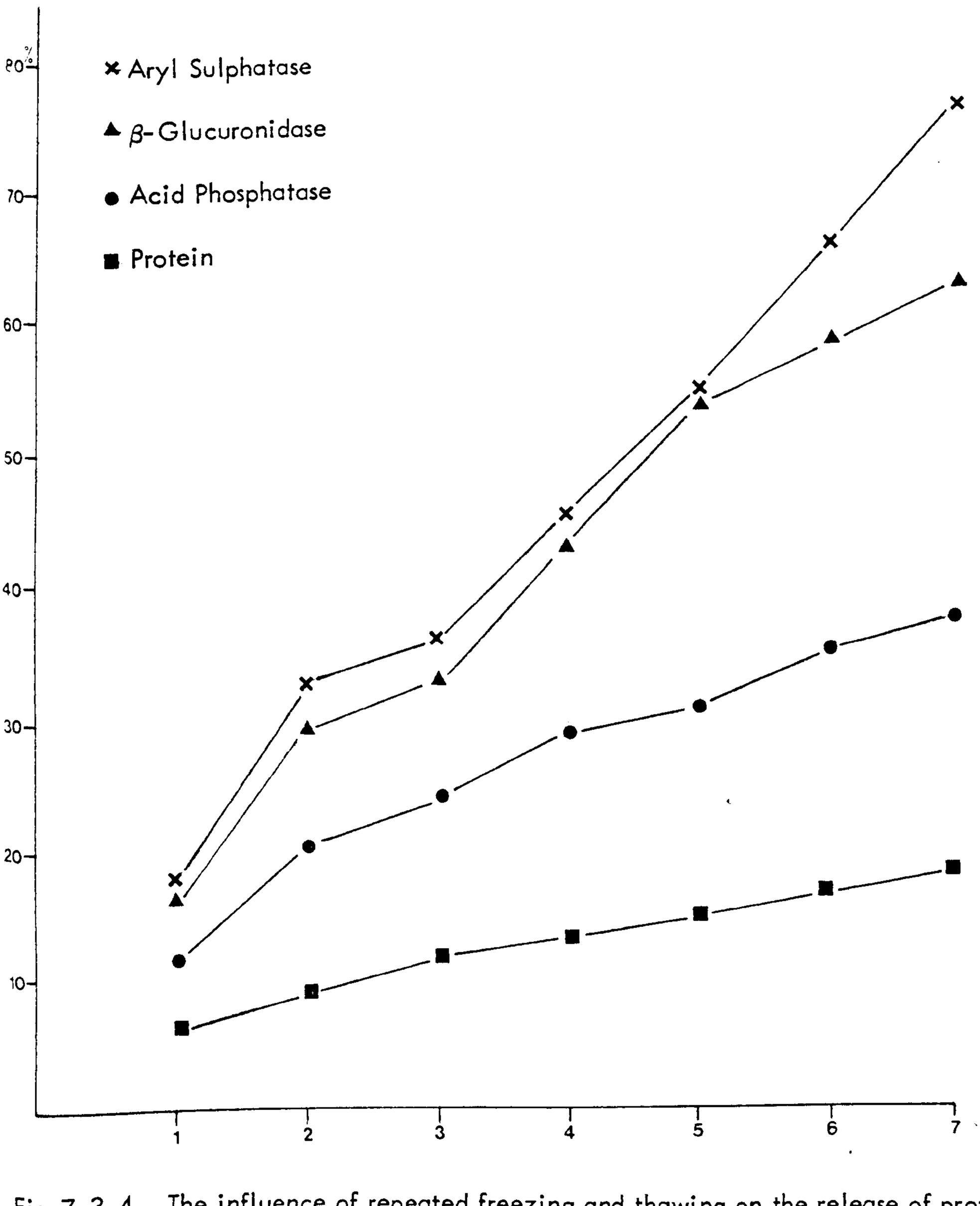


Fig.7.3.4. The influence of repeated freezing and thawing on the release of protein and enzyme from a dog urinary bladder lysosome preparation

Table 7.3.6. The effect of repeated freezing and thawing on the release of protein and enzymes from a lysosomal preparation from dog urinary bladder cells

Number of freezing and thawing cycles	Protein			β-Glucuronidase			Acid Phosphatase			Aryl Sulphatase		
	µg/ml present	µg/ml released	% of bound released	units/ml present	units/ml released	% of bound released	units/ml present	units/ml released	% of bound released	units/ml present	units/ml released	% of bo release
0	51	-	-	0.9	-	-	15.5	-	-	0.33	-	-
1	56	5	6	2.8	1.9	16.0	25.5	10	10.8	1.07	0.74	17.3
2	59	8	9	4.4	3.5	29.4	34.5	19	20.5	1.73	1.40	32.8
3	61	10	12	4.8	3.9	32.8	37.5	22	23.8	1.87	1.54	36.1
4	62	11	13	6.0	5.1	42.8	42	26.5	28.7	2.26	1.93	45.3
5	64	13	16	7.5	6.6	55.5	44	28.5	30.8	2.73	2.40	56.3
6	65	14	17	8.0	7.1	59.6	48.5	33	35.7	3.20	2.87	67.4
7	67	16	19	8.5	7.6	63.9	50	34.5	37.3	3.64	3.31	77.6
Total activity	134			12.8			108			4.60		
Bound activity	83			11.9			92.5			4.27		

lysosome- rich fraction has been prepared from dog urinary bladder epithelial cells, and that experiments on the effects of amines and their derivatives are therefore possible.

In order to carry out such studies it was necessary to add the amines in a solvent which would not have a greater lytic effect on the organelles than that observed as spontaneous release during incubation under test conditions. The results obtained on the effects of several solvents are shown in Table 7.3.7.

Table 7.3.7 The influence of solvents on the release of enzymes from a dog urinary bladder preparation after incubation at 37° for 1h.

Addition (Final concentration 1%)	Percentage of Bound Enzyme Released			
	β-Glucuronidase	Aryl Sulphatase	Acid Phosphatase	Cathepsin
Nil	16	12	22	8
Ethanol	22	20	30	15
Acetone	16	15	20	12
Dimethylsulphoxide	16	12	20	9

The solvents were chosen because of their demonstrated ability to dissolve the test compounds at the required concentration. Each of the enzymes studied was released to a greater extent in the presence of ethanol 0.1% than in control incubates. In view of the minimal effects observed with dimethylsulphoxide this solvent was used in subsequent experiments.

During the course of these experiments, designed to verify the possibility of isolating a fraction containing "bound" enzymes within an organelle, it became apparent that considerable variation, both in the sub-cellular distribution and

the stability of the enzymes within the organelles during subsequent incubation at 37,° existed. This variation could have resulted from the method of preparation or could have occurred naturally depending upon the physiological status of the animal. As this variation in fragility could make difficult a comparison of effects of exogenous materials on lysosomal stability, it was decided to establish an arbitrary measure of fragility. This was done as described in 7.2.4.8, and fragility was defined as the percentage of bound β-glucuronidase which becomes non-sedimentable under the conditions used, after incubation for 1 h at 37° in the presence of 1% dimethylsulphoxide. In practice most preparations had a "fragility" between 15 and 30%; any preparation falling outside this range was discarded and not used for further work in Section 7.2.5.

In order to determine whether aromatic amines, or their hydroxylated derivatives, were able to interact with lysosomes and so release bound enzymes, experiments were carried out in which the test compounds were incubated with lysosomal preparations at 37° for 1 h. Because of the variations in the characteristics of the preparation, both in regard to enzyme content and to fragility, it was impossible to obtain an absolute measure of effect. Instead the effect was measured by a comparison of the enzyme activity released in the presence of the test compound compared to the amount of enzyme released spontaneously under the same test conditions.

At the same time, it was important to measure the effect of the test compounds on the enzyme activity being measured. A situation could exist where the organelle was lysed yet because of the inhibitory action of the lysing agent on the enzyme measured no apparent lysis was observed. In order to test this possibility each test mixture was also run in the presence of Triton X-100.

The results obtained from a series of amines, and their hydroxylated derivatives, on the release of β-glucuronidase from a lysosomal preparation are tabulated in Table 7.3.8. From this table several interesting conclusions can be drawn. The first is that the majority of parent amines studied have little effect on lysosomal stability, as figures between 0.8 and 1.1 indicate. However, 4-hydroxylaminobiphenyl does consistently release high levels of β-glucuronidase compared to lysosomal incubate without this addition, or compared with 4-aminobiphenyl, 4-amino-3-hydroxybiphenyl or 4-amino-4'-hydroxybiphenyl. In contrast, certain compounds appear to exert a protective effect on the lysosomal membrane preventing the release of β-glucuronidase. This seems particularly true of the hydroxylated naphthylamines, yet when the effect of these compounds on β-glucuronidase activity was studied (Table 7.3.8) it can be seen that it was these compounds which had the greatest inhibitory effect. As the relative concentration of these compounds would be correspondingly higher when acting only on spontaneously released enzyme, inhibition rather than stabilisation may account for this phenomenon. Amino-naphthols are easily oxidised at pH 7.4 (Gorrod, Alifano, Papa & Quagliarello, 1967) to yield a variety of products (Nagasawa, Gutmann & Morgan, 1959). The parent substances or the oxidation products have the ability to covalently bind with proteins (Bellman & Troll, 1962; Nagasawa & Gutmann, 1959). Visual examination of the sediment from lysosomal experiments in the presence of amino naphthols indicated that binding to a cellular constituent had occurred as the material was stained with a blue pigment.

Table 7.3.8. The effect of aromatic amines and their hydroxylated derivatives on the release of sedimentable β-glucuronidase obtained from dog urinary bladder epithelial cells

Compound[+]	Effect on release[†]	% Activity of enzyme[×]
Aniline	0.9	98
Phenylhydroxylamine	0.9	94
Ortho-aminophenol	0.7	99
Para-aminophenol	0.5	98
Anthranilic acid	0.9	96
Ortho-carboxyphenylhydroxylamine	0.9	102
3-Hydroxyanthranilic acid	0.7	99
5-Hydroxyanthranilic acid	0.6	97
1-Naphthylamine	0.8	99
1-Naphthylhydroxylamine	0.9	98
1-Amino-2-naphthol	0.2	80
1-Amino-4-naphthol	0.1	76
2-Naphthylamine	0.8	110
2-Naphthylhydroxylamine	1.1	100
2-Amino-1-naphthol	0.4	80
2-Amino-5-naphthol	0.4	82
4-Aminobiphenyl	1.4	105
4-Hydroxylaminobiphenyl	2.6	95
4-Amino-3-hydroxybiphenyl	0.9	92
4-Amino-4'-hydroxybiphenyl	0.9	100

[+]Compounds present at a concentration of 0.5 mM.

[†]Effect = $\frac{\beta\text{-glucuronidase activity released in presence of compound}}{\beta\text{-glucuronidase activity released in absence of compound.}}$

[×]β-Glucuronidase activity measured in the presence of compounds (0.5mM) and Triton X-100 (0.1%).

As 4-hydroxylaminobiphenyl had the greatest effect on release of β-glucuronidase, further experiments were carried out with increasing concentrations of certain arylhydroxylamines. In each case the maximum measurable effect occurred at a concentration of 1 mM (Table 7.3.9).

Table 7.3.9. The effect of increasing concentration of arylhydroxylamines on the release of β-glucuronidase from dog urinary bladder lysosomes

Compound	Concentration (mM)	Effect on enzyme release[+]	Effect on enzyme activity[x]
Phenyl-hydroxylamine	0.5	1.1	98 %
	1.0	1.7	82
	2.0	1.3	75
Orthocarboxyphenyl-hydroxylamine	0.5	0.9	100
	1.0	1.1	92
	2.0	0.3	73
2-Naphthyl-hydroxylamine	0.5	1.1	98
	1.0	1.3	82
	2.0	0.8	69
4-Hydroxylamino-biphenyl	0.5	1.7	98
	1.0	1.8	86
	2.0	1.4	62

[+]Effect = $\frac{\beta\text{-glucuronidase activity released in presence of compound}}{\beta\text{-glucuronidase activity released in absence of compound}}$

[x]β-Glucuronidase activity measured in the presence of compounds and Triton X-100 (0.1%)

Higher concentrations apparently had less effect. However, as the increased concentrations of the hydroxylamines all caused a greater inhibition of β-glucuronidase, this may have produced an erroneous picture of the situation.

In a further experiment 4-aminobiphenyl and some related hydroxylated compounds were examined for a lytic effect with regard to the release of aryl sulphatase and acid phosphatase from a dog urinary bladder lysosomal preparation. A strong lytic activity with regard to aryl sulphatase was produced (Table 7.3.10) with 4-hydroxylaminobiphenyl, but not with regard to acid phosphatase.

Table 7.3.10 The effect of 4-aminobiphenyl and some related hydroxylated derivatives on the release of acid phosphatase and aryl sulphatase from a dog urinary bladder lysosomal preparation

Compound (0.5 mM)	Acid Phosphatase		Aryl Sulphatase	
	Effect*	Activity+	Effect*	Activity+
4-Aminobiphenyl	2.0	83	1.5	100
4-Hydroxylaminobiphenyl	1.8	95	3.0	116
4-Amino-3-hydroxy-biphenyl	1.6	102	1.0	120
4-Amino-4'-hydroxy-biphenyl	-	72	0.3	62

*Effect = $\frac{\text{Enzyme activity released in presence of compound}}{\text{Enzyme activity released in absence of compound}}$

+Enzyme activity measured in the presence of compounds (0.5mM) and Triton X-100 (0.1%).

In these experiments it was found that 4-amino-4'-hydroxybiphenyl was an inhibitor of both acid phosphatase and aryl sulphatase, whereas an activating influence was seen on aryl sulphatase by both 4-hydroxylamino-biphenyl and 4-amino-3-hydroxybiphenyl.

It is clear from these experiments that no conclusive correlation has been found between any structurally similar group of compounds and their effect on β-glucuronidase release from dog bladder lysosomes. The finding that aryl-hydroxylamines released β-glucuronidase, although not with the same efficiency, certainly supports Allison's theory of carcinogenesis by aromatic amines. However, when the release of aryl sulphatase and acid phosphatase was examined, only the former enzyme was released to an appreciable extent by 4-hydroxylaminobiphenyl compared with the other compounds studied. Several reasons for this phenomenon can be postulated.

It is now realised that the lysosome population within a cell is very heterogeneous (Section 7.1), and in experiments of this type only those lysosomes which survive the isolation procedure are available for study. As considerable amounts of lysosomal enzymes were detected in the supernatant (Table 7.3.1), it may be that the lysosomes most sensitive to the carcinogenic (or indeed any other) stimuli, were already destroyed. Further, it is well documented (Tappel, 1969), and supported by work in this thesis, that different enzymes do not become released from the lysosome matrix at the same rate.

Extending the study to other enzymes, therefore, may have afforded a better correlation between carcinogenic activity and lysosomal damage. Such a project is fraught with difficulty, even if sufficient tissue were to become available,

because many of the compounds of interest interfere with the assay methods used for determining enzymic activity. For example, hydroxylated compounds react with Folin-Ciocalteau reagent used in the assay of cathepsin, and the aromatic amines and their hydroxylated derivatives strongly absorb in the shorter wavelengths commonly used for the assay of both ribonuclease and desoxyribonuclease.

Another aspect of this problem is one of experimental design. Because of the practical problems in working with dog urinary bladder tissue, a system was developed which examined the release of enzymes from the lysosomes due to external stimuli. It is worth remembering that under the experimental conditions whereby bladder tumours are produced from aromatic amines, the amines and their metabolites are external to the epithelial cells, but these may be encapsulated inside the organelle by pinocytosis. In order to carry out this type of experiment measurement of "free" and "bound" enzymes in susceptible cells during amine feeding experiments, would have to be carried out. Clearly this experiment was not feasible using dogs and aromatic amines as the test system. Whilst the test system developed herein is concerned with the leakage of enzymes out of the organelle, another aspect which was not explored was the possibility of the test compounds altering the permeability of the membranes, and allowing substrates to enter the organelle. This was not considered further as Allison's concept recognised that the nuclease had to get to nuclear nucleic acids rather than vice-versa.

At present the mechanism by which 4-hydroxylaminobiphenyl allows excess leakage of β-glucuronidase and aryl sulphatase from the organelle is not known.

It has been proposed that a peroxidation reaction involving the membrane is a prerequisite for enzyme release (Wills & Wilkinson, 1966). In experiments in which methyllinoleate was used as a model system, 4-hydroxylaminobiphenyl has failed to enhance the formation of malonaldehyde, indicating that lipid peroxidation is not affected by this compound.

A similar release of β-glucuronidase from a rabbit liver lysosome preparation has been reported with N-hydroxy-2-acetamidofluorene (1mM), the parent amide being inactive (Weissmann, Troll, van Duuren & Sessa, 1968). In a more recent paper, Berg and Christoffersen (1974) have shown that a considerable redistribution of hepatic lysosomal enzymes occurs after feeding 2-acetamidofluorene to rats, they suggest that larger lysosomes are formed indicative of increased cellular autophagy. This biochemical study is consistent with the findings of Flaks (1970) referred to earlier. However, work by Takano, Kato, Miyata, Goto, Ohkuma, Mizuno, Kitigawa & Yokoyama (1971), on the distribution of hepatic lysosomal enzymes during carcinogenesis experiments with dimethylaminoazobenzene, showed an increase in the amounts of β-glucuronidase and acid ribonuclease present in the cytoplasm and less in the particulate form, indicating to the authors a change in lysosomal stability during carcinogenesis.

The results in this section do not invalidate the possible role of lysosomal damage in carcinogenesis proposed by Allison (1967, 1969). The results do show the extreme complexity of the present state of our knowledge on lysosomes, and the difficulties involved in working with experimental systems directly related to the disease induced in man.

SECTION 8 THE ACTIVATION OF β-GLUCURONIDASE BY CERTAIN AROMATIC AMINES.

8.1 INTRODUCTION

The enzyme β-glucuronidase is of considerable importance. Its localisation within the lysosome (see Section 7) has allowed its widespread use in tissue fractionation studies (De Duve, Pressman, Gianetto, Wattiaux & Applemans, 1955; Wattiaux, 1969; Dingle & Fell, 1969; Dingle, 1972) as a marker enzyme. Ganschow & Paigen (1967) have found differences in the properties of the enzyme derived from different organelles within cells, and have indicated that as these properties are under a genetic control mechanism, β-glucuronidase can be used as a suitable genetic marker.

β-Glucuronidase is present in plasma, milk, vaginal fluid and urine of mammals, and its activity is modified during many physiological processes, e.g. pregnancy, (Woessner, 1965), involution of the mammary gland and during the menstrual cycle (Helminen & Ericsson, 1968). Elevated levels of urinary β-glucuronidase have been associated with cancer of the urinary tract in humans (Boyland, Wallace & Williams, 1955; Rappappert & Richterich, 1965; Mattea & Pietra, 1959; Abul-Fadl, 1957), and its estimation in urine has been suggested to be of value for prognostic purposes in this disease. Bank & Bailine (1965) reported that urinary β-glucuronidase was increased in patients with acute or chronic pyelonephritis compared with those patients having infections of the lower urinary tract. These latter conclusions have recently been questioned by Roberts, Frampton, Karim and Beard (1967) who found elevated levels of the enzyme in a number of normal subjects with sterile urine. These latter authors also found elevated urinary β-glucuronidase during gestation in humans.

Urinary β-glucuronidase has also been reported to be elevated in animals receiving either hepatotoxic or nephrotoxic agents (Soloimskaya, 1970), and it has been suggested that the increase in enzyme level may be used as an index of tissue damage (Slater, 1966).

In view of the known excretion of metabolites of 2-naphthylamine as conjugates with glucuronic acid, Boyland (1956) implicated β-glucuronidase in the etiology of bladder cancer. Boyland (1956) suggested that β-glucuronidase present in urine was able to hydrolyse 2-amino-1-naphthyl-β-glucosiduronic acid to release 2-amino-1-naphthol as a locally-acting bladder carcinogen. Boyland and Williams (1960) showed that β-glucuronidase from a variety of sources was able to hydrolyse 2-amino-1-naphthol-β-glucosiduronate, and suggested that inhibition of the enzyme may be of value in the treatment of cancer of the urinary tract. Attempts at chemotherapy using this approach have been carried out (Boyland, Kinder, Manson & Wallace, 1965; Boyland, Wallace & Williams, 1957), but protection against bladder tumour formation has not been obtained. This may have been due to the rapid metabolism of the inhibitor glucosaccharo-1→4-lactone, resulting in a high level of residual enzyme activity present in the urine.

However, Miyakawa, Yoshida, Harada & Kato (1973) did find apparent suppression of bladder tumour formation by treatment of rats receiving 2-acetamidofluorene, with the potent β-glucuronidase inhibitor 2,5 di-O-acetyl-D-glucosaccharo-1→4 : 6→3 - dilactone.

During the course of the experiments described in section 7, a small but constant activation of β-glucuronidase was observed in the presence of

certain aromatic amines. This activation was not due to the release of the enzyme from an organelle, as activation was observed in preparations which had been treated with the non-ionic detergent Triton X-100, which is known to solubilise β-glucuronidase (Wattiaux & De Duve, 1956).

In view of the widespread measurement of β-glucuronidase activity for monitoring purposes, it would seem that any factors altering the activity of this enzyme could produce misleading results unless they were taken into consideration. For these reasons some further experiments on the activation of β-glucuronidase by aromatic amines have been undertaken.

8.2 EXPERIMENTAL

8.2.1 Chemicals

Aniline, ortho-, meta- and para-toluidines, mesidine, anthranilic acid and para-aminobenzoic acid were obtained from Hopkins & Williams; 4-aminobiphenyl, 2-naphthylamine, 2-aminochrysene, benzidine, 2,7-diaminofluorene, 2-aminofluorene from Koch Light Laboratories, and 1-naphthylamine from British Drug Houses. 6-Aminochrysene and 3-aminopyrene were gifts from Dr.P.Sims, and 4'-fluoro-4-aminobiphenyl, 2-aminobiphenylene oxide and glucuronic acid were gifts from Dr.D.Manson, both of the Chester Beatty Research Institute. All chemicals were purified by recrystallisation or distillation until only one substance was detected when examined by chromatographic methods.

8.2.2 Enzyme

Purified beef liver β-glucuronidase (Ketodase, Warner-Chilcot) was used throughout this section; dilutions of the enzyme were carried out using 0.2M

acetate buffer, pH 4.6, and compounds were incorporated as solutions in dimethylsulphoxide (DMSO) (B.D.H.) (final concentration 1%).

8.2.3 Assay Procedure

β-Glucuronidase activity was assayed by the method of Talalay, Fishman & Huggins (1946) by following the release of phenolphthalein from phenolphthalein mono-β-D-glucuronide (1mM) as described in Section 7.

8.2.4 Specificity of amine activation of β-glucuronidase

Aromatic amines were incorporated into the β-glucuronidase substrate mixture at a final concentration of 0.25 mM. The enzyme was diluted to have an activity of between 0.5 and 0.7. Fishman units and was assayed by incubation in the presence of the amines for 15 h at 37° at pH 4.6.

8.2.5 Effect of amine concentration on the activation of β-glucuronidase

Aniline, 4-aminobiphenyl, 2-aminofluorene and 2-aminobiphenylene oxide were examined as above, except that the concentration of the amine was varied between 0.25 mM and 2.0 mM. The concentration of the solvent (DMSO) was kept constant at 1%.

8.2.6 The influence of D-glucuronic acid on the activation of β-glucuronidase by aromatic amines

Certain aromatic amines, at a concentration of 0.25 mM, were pretreated with D-glucuronic acid (0.5mM) at pH 4.6 for 20 h at 37° prior to the addition of enzyme and substrate. The enzyme activity was then assayed as above.

8.2.7 The influence of time after dilution on the activation of β-glucuronidase by aromatic amines

β-Glucuronidase was diluted as before to give a solution containing approximately 0.7 Fishman units. The solution was immediately divided and portions

treated with aromatic amines at a final concentration of 0.5 mM. One portion untreated with amine served as a control. All the experiments were carried out at 37° and pH 4.6. Aliquots were removed at various time intervals and assayed as before.

8.2.8 Reactivation of β-glucuronidase by aromatic amines

A diluted (0.7 Fishman units) solution of the enzyme was stored at 0-3° for 10 h , following which certain aromatic amines at a final concentration of 0.5 mM were added to portions of the enzyme, and the mixture again stored for 10 h at 0-3°. The enzyme activity was then determined as before. In a further experiment aliquots of reaction mixture were removed at 0, 1, 2, 4, 7 and 10 h after the addition of the amines to the diluted and stored enzyme and assayed as described before.

8.3 RESULTS AND DISCUSSION

The specificity of the activation of β-glucuronidase is indicated in Table 8.3.1. In experiments at two enzyme concentrations no real evidence of activation can be seen after incorporation of the monocyclic aromatic amines used. Activation was observed with the bicyclic aromatic mono amines studied except 1-naphthylamine, but the activation was lost when the amino group was associated with a larger polycyclic aromatic ring system. It is of interest that 2-aminobiphenylene oxide, which contains a hetero-oxygen ring system, was the most potent activator found in the series studied. It may be that certain steric requirements are essential for activation, and it is tempting to suggest that substitution in the para-position is a prerequisite. Whilst this could account

Table 8.3.1. The effect of various aromatic amines on the activity of a dilute beef liver β-glucuronidase preparation

Amine present§	Phenolphthalein formed (μg)*		Phenolphthalein formed (μg)*		Mean activity
	Expt.1	% activity	Expt.2	% activity	
No addition	10.2	100	6.5	100	100.0
Aniline	10.5	103	7.0	108	105.5
Ortho-Toluidine	9.6	94	6.3	97	95.5
Meta-Toluidine	10.2	100	6.8	104	102.0
Para-Toluidine	10.0	98	6.4	98	98.0
Mesidine	10.2	100	6.6	101	100.5
Anthranilic Acid	9.8	96	6.4	98	97.0
Para-Aminobenzoic Acid	10.4	102	6.8	104	103.0
1-Naphthylamine	10.5	103	7.0	108	105.5
2-Naphthylamine	11.8	116	8.5	130	123.0
4-Aminobiphenyl	11.4	112	8.0	123	117.5
4'-Fluoro-4-aminobiphenyl	11.4	112	8.8	135	123.5
2-Aminofluorene	11.2	110	8.0	123	116.5
2-Aminobiphenylene oxide	13.0	127	10.1	155	141.0
3-Aminopyrene	10.0	98	6.6	101	99.5
2-Aminochrysene	10.5	103	6.8	104	103.5
6-Aminochrysene	10.0	98	6.5	100	99.0
4,4'-Diaminobiphenyl	6.0	59	4.5	69	64.0
2,7-Diaminofluorene	6.2	61	4.5	69	65.0

§ All amines were present at a concentration of 0.25 mM.

* During 15 h incubation at 37° from phenolphthalein-β-D-glucuronide (1.0 mM).

for the lack of activity of 1-naphthylamine, compared to the 2-isomer and 2-aminochrysene, it does not explain the inactivity of 6-aminochrysene, 2-aminopyrene, para-toluidine or para-aminobenzoic acid, all of which have para substituents.

Neither is there any correlation between ability to active β-glucuronidase and published pKa values for the amines studied. Aniline, pKa 4.59, was inactive, whereas 2-aminofluorene, pKa 4.64, was active. Additionally, the inactive amine 1-naphthylamine (pKa 3.92) is less basic than the active 2-isomer (pKa 4.11), whereas para-toluidine is more basic (pKa 5.12) and yet remains inactive. If, as discussed later, a surface effect is operating in this activation process, then the solubilities or partition coefficients may give the desired correlations. Both of the diamines studied showed marked inhibitory effects on the enzyme and were not studied further. The influence of amine concentration is shown in Table 8.3.2.

In the case of the three active amines an increase in activating ability with increased concentration is shown. However, there seems to be a limiting value at about 0.5 to 1 mM, as doubling the concentration to 2 mM does not further enhance the amount of hydrolysis. These results also confirm the inability of aniline to activate β-glucuronidase, even when the concentration is raised to 2 mM.

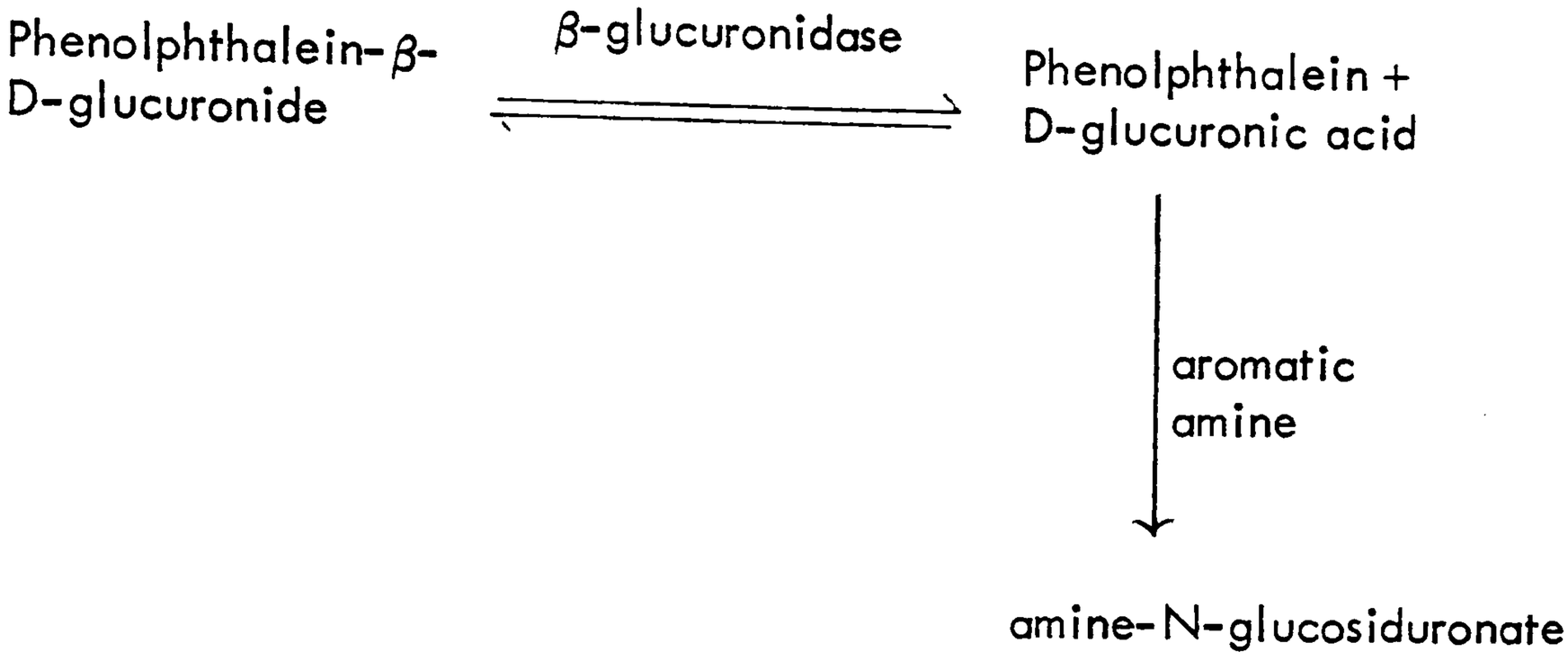
The experiments where the aromatic amines were pretreated with D-glucuronic acid were performed to ascertain whether a spontaneous reaction between the amine and D-glucuronic acid, released from phenolphthalein-β-

Table 8.3.2. Influence of concentration on the activation of β-glucuronidase by aromatic amines

Amines	Concentration mM	Phenolphthalein liberated (μg)*	Activity
No addition	-	4.2	100
Aniline	0.25	4.2	100
"	0.5	3.8	91
"	1.0	4.0	95
"	2.0	4.2	100
4-Aminobiphenyl	0.25	5.0	119
"	0.5	6.1	145
"	1.0	7.2	171
"	2.0	7.5	179
2-Aminofluorene	0.25	6.2	148
"	0.5	7.0	167
"	1.0	8.2	195
"	2.0	8.5	202
2-Aminobiphenylene oxide	0.25	7.2	145
"	0.5	8.0	190
"	1.0	8.2	195
"	2.0	8.5	202

* during 15 hrs incubation from phenolphthalein-β-D-glucuronide (1.0 mM) at 37°, pH 4.6.

D-glucuronide, could occur and cause a shift in the equilibrium, viz:



Such reactions between aromatic amines and D-glucuronic acid are known to occur (Boyland, Manson & Orr, 1957; Bridges, 1964; Gorrod, 1960) and could account for the activation observed. The results in Table 8.3.3 indicate that this is not the case, as active amines still show the same activation of β-glucuronidase even after pretreatment with D-glucuronic acid. Under these conditions, D-glucuronic acid was without effect on the control enzyme activity in the presence or absence of aniline and did not affect the slight activation effect of 1-naphthylamine seen in this experiment.

During the course of the previous work in this section it became apparent that some differences in magnitude of activation under the influence of a fixed concentration of amine occurs. This is apparent from the two experiments shown in Table 8.3.1 where the same enzyme was used but different times elapsed between dilution of enzyme and its activation by amines. It was therefore, thought desirable to examine the influence of time after dilution of the enzyme on the subsequent effect of amines on enzyme activity. The results are shown in Fig.8.3.1. With no addition of amine, and under the conditions employed, a loss of activity occurs which is rapid during the first few hours after enzyme

Table 8.3.3 The effect of pretreating aromatic amines with D-glucuronic acid on the activation of β-glucuronidase

Amine Present*	Phenolphthalein liberated§ (μg)	
	No pretreatment	Pretreatment with Glucuronic acid+
No addition	6.9	6.7
Aniline	6.7	6.5
4-Aminobiphenyl	8.2	8.2
4'-Fluoro-4-aminobiphenyl	8.8	8.4
2-Aminofluorene	8.0	8.0
2-Aminobiphenyleneoxide	10.2	10.2
2-Naphthylamine	8.5	8.3
1-Naphthylamine	7.2	7.2

* Amines present at final concentration of 0.25 mM.

\+ Amines were treated with glucuronic acid 0.5 mM at pH 4.6 and 37° for 20 h prior to the addition of the enzyme and substrate.

§ During 15 h incubation at 37° from phenolphthalein-β-D-glucuronide (1 mM) at pH 4.6.

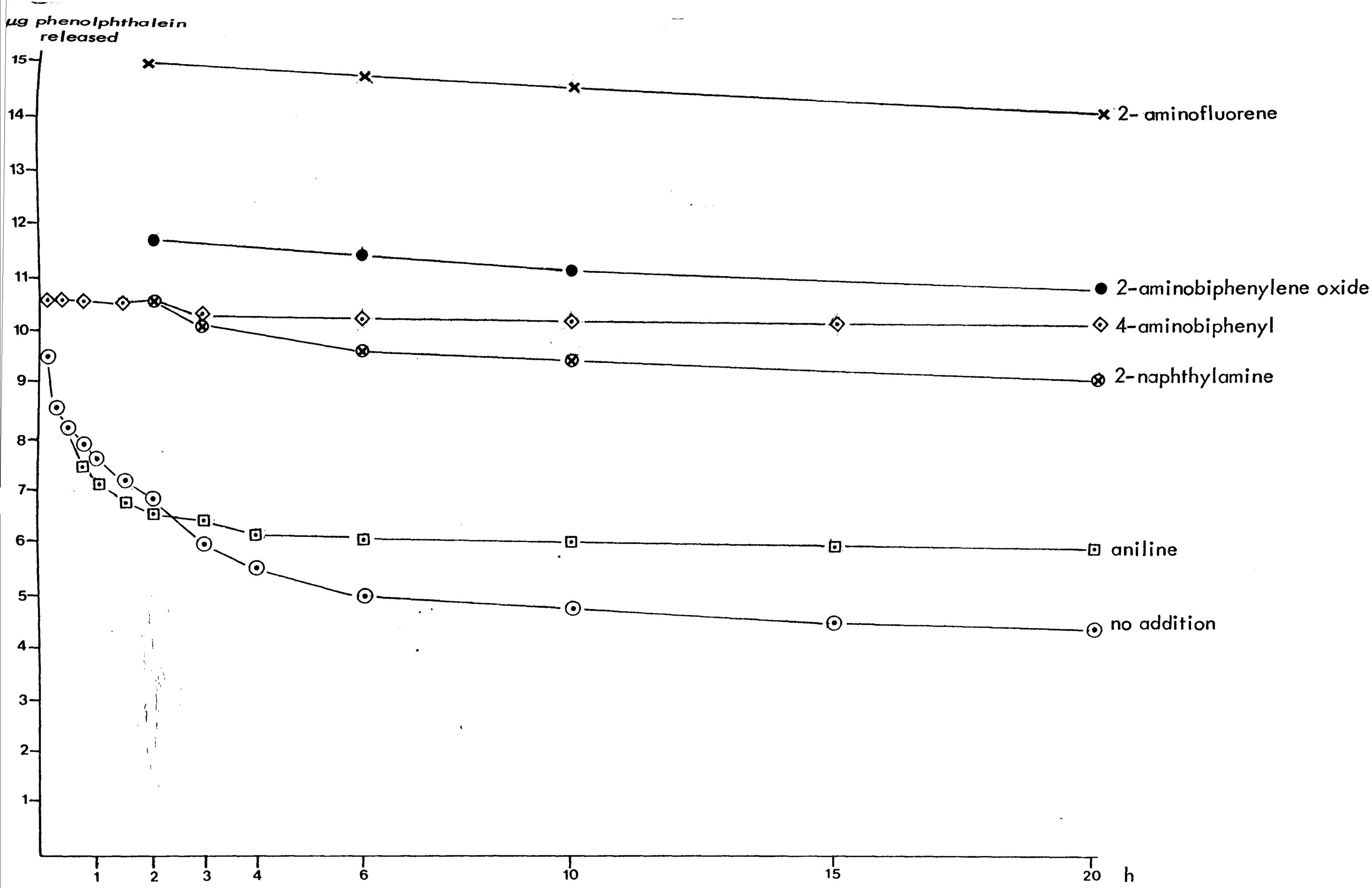


Fig.8.3.1. The Influence of certain aromatic amines on the activity of the enzyme β-Glucuronidase at different times after dilution

dilution, but does not decrease appreciably after ten hours. In the presence of aniline the rapid decay is again observed, but, in this case, the plateau level of enzyme activity is maintained after four hours. The other aromatic amines studied completely prevent the loss of activity observed in their absence. In the case of both 2-aminofluorene and 2-aminobiphenylene oxide considerably higher levels of released phenolphthalein were obtained compared with the control preparations. This suggests that on dilution a very rapid dissociation of the enzyme occurs with a concomitant loss of enzyme activity. The aromatic amines could be producing their apparent activation effect by preventing this dissociation and the level of "activation" observed would then depend on their ability to prevent dissociation. If this is true, then the rate of dissociation must either be very rapid during the first few minutes after dilution or certain amines must have the ability to enable the enzyme sub-units to reassociate to form more of the active species.

In an attempt to see whether the latter postulate is tenable a solution of the diluted enzyme was stored at 0-3^{o} for 10 h to allow inactivation (dissociation) to occur. After a further period of 10 h in the presence of amine, to allow reassociation, the enzyme activity was again measured and is shown in Table 8.3.4. In every case the activity of the stored diluted enzyme was enhanced, aniline having the smallest effect and 2-aminofluorene the greatest, with the other amines producing an intermediate effect. The reactivation of the diluted enzyme by amines appeared to be related to the protective effect shown in Fig.8.3.1. An attempt to show a gradual reactivation of diluted stored enzyme by certain amines with time failed, as all the amines studied, except aniline, produced an activation of enzyme activity even when the enzyme assay was

Table 8.3.4. The reactivation of β-glucuronidase* by aromatic amines.

Amine (0.5 mM)	μg phenolphthalein §
No addition	3.0
aniline	3.9
4-aminobiphenyl	5.0
4'-fluoro-4-aminobiphenyl	6.9
2-naphthylamine	5.0
2-aminofluorene	8.5
2-aminobiphenylene oxide	7.2

* Enzyme diluted and stored at 0-3° for 10 h prior to addition of amine, then stored for a further 10 h

§ Liberated during 15 h incubation at 37°.

initiated immediately after the addition of the amine. In this latter experiment it may have been that the long incubation period of the assay, due to the low concentration of the enzyme, masked the gradual reactivation hoped for (Fig. 8.3.2). In fact, the continual decrease in activity shown by the enzyme either in the presence or absence of aniline, clearly indicates that the enzyme activity had not plateaued at the ten hours chosen for storage in this experiment.

The indication, found in Section 7, that certain aromatic amines caused an activation of β-glucuronidase, has been confirmed in this wider study. It has also been confirmed that the activation, when carried out in the presence of concentrated, fresh enzyme preparations, is only slight. This activation can be highly significant if determined using aged diluted enzyme solutions. The results suggest that the apparent activation may be caused by preventing the inactivation produced by dilution and/or storage, rather than a true activation.

The dissociation of β-glucuronidase was first recognised by Bernfeld, Bernfeld, Nisselbaum and Fishman (1954). These authors indicated that β-glucuronidase formed inactive subunits upon dilution, but that the activity could be retained in the presence of certain macromolecules, e.g. bovine serum albumin, desoxyribonucleic acid from various sources, gelatin, chymotrypsin, ribonucleic acid and certain α, ω-diaminomethylenes. Under the conditions used by these authors, at least two basic groups per molecule were required to produce an "activator". Although activating ability was found in all members of the series of diamines $NH_2(CH_2)_nNH_2$, maximal activation was observed in the diamine $NH_2(CH_2)_{10}NH_2$. In contrast the results reported here show that the two aromatic amines studied benzidine and 2,7-diaminofluorene did not activate the enzyme but actually caused considerable inhibition of enzyme activity.

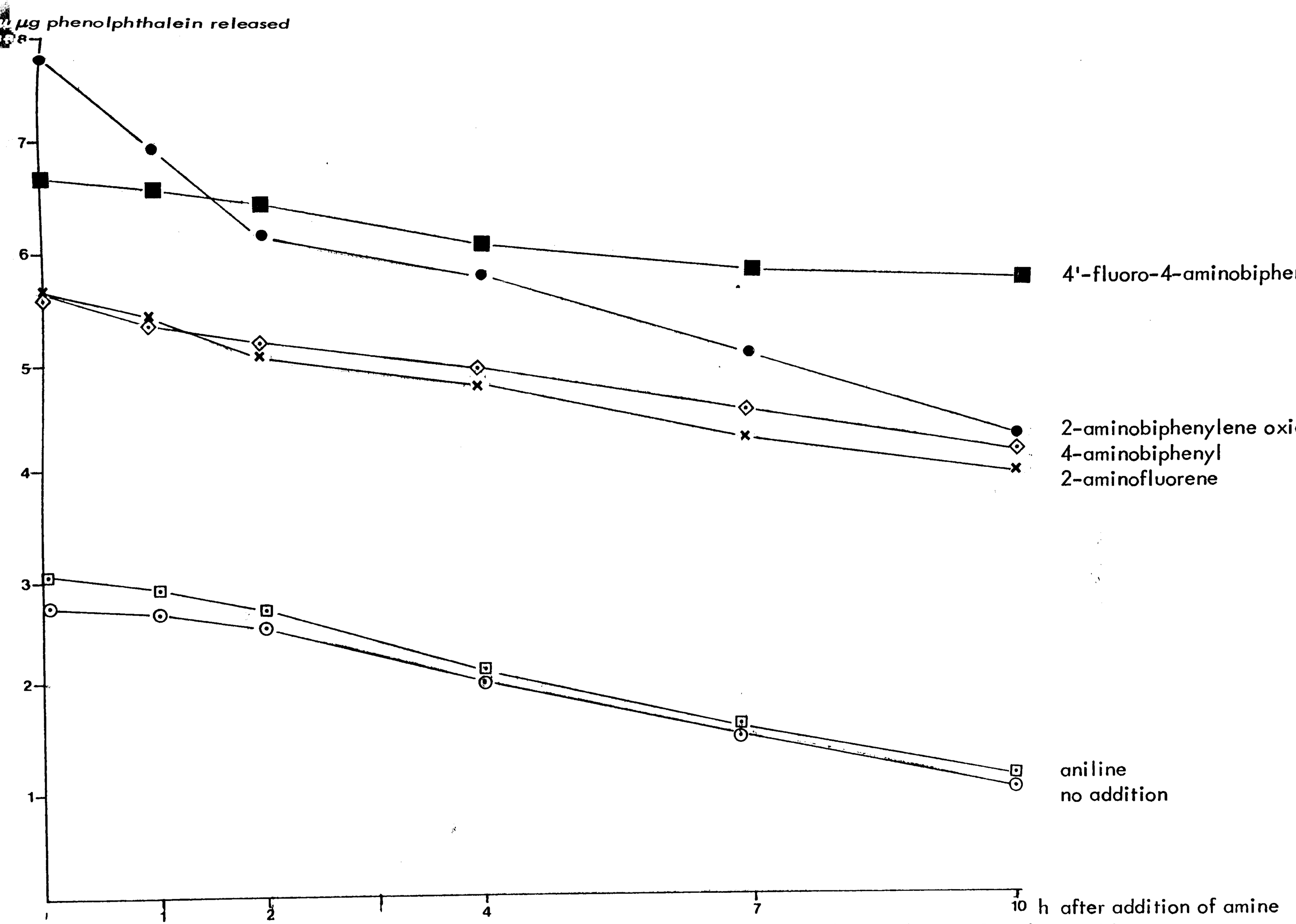


Fig.8.3.2. The influence of certain aromatic amines on the activity of a stored diluted solution of β-glucuronidase

Bernfeld, Jacobson and Bernfeld (1957) confirmed the reversible nature of the dissociation occurring upon dilution of β-glucuronidase, and its "activation" by macromolecular polycations. They also showed that this "activation"could be prevented by macromolecular polyanions, presumably by reaction with the polycation. In this paper a further slow inactivation of the dissociated sub-units is shown to occur if they are exposed to rough surfaces such as silica,sand or glass powder, after which reactivation by polyamines is unable to occur. A further type of activation of β-glucuronidase was reported in an "Urgent Bulletin on Bacterial β-Glucuronidase", issued by Sigma Chemical Co.(1958), in which chloroform, often used as a preservative in biological samples, had the ability to increase the activity of β-glucuronidase by up to seven hundred per cent. This activation was confirmed by Gautney, Barker & Hill (1959) who showed that several aliphatic alcohols, as well as halogenated hydrocarbon solvents, also had this activation effect upon the enzyme derived from bacterial systems, but not on the mammalian enzyme.

The degree of activation of the bacterial enzyme system by chloroform has been shown by Ryan and Mavrides (1960) to depend upon the area of the chloroform/aqueous interface. It would seem unlikely that a surface phenomenon is occurring in the experiments described in this section. In the cases where it was seen that the amines were not wholly in solution, i.e. 3-aminopyrene or the aminochrysenes, activation was not observed (Table 8.3.1). It would seem that the prevention of dissociation of the enzyme by aromatic amines is more likely to be the mechanism involved. Recently it has been shown that lysosomal β-glucuronidase from rat liver is a tetramer having a molecular weight of approximately 280,000, which under certain conditions dissociates

into subunits of about 75,000 (Stahl & Toaster, 1971). In order to confirm the "protection" hypothesis it would be interesting to carry out experiments using either ultracentrifugation or gel electrophoretic techniques in the absence and presence of "activators".

Since the completion of this work, my attention has been drawn to a report by Takanashi, Ohkubo, Takahashi, Ida, Okutomi & Kawada (1964) in which they perform some experiments on N-glucuronide formation. In these experiments β-glucuronidase is used as a transferase to transfer the glucuronic acid from phenolphthalein -β-glucuronide to 2-naphthylamine. These authors found that the amount of phenolphthalein formed was increased with increasing concentration of 2-naphthylamine and concluded "that β-glucuronidase is somewhat activated in the presence of 2-naphthylamine". The results in this section confirm and extend their conclusions.

In view of the known elevated concentrations of endogenous aromatic amines in certain pathological conditions (see Section 1), it is suggested that the raised levels of β-glucuronidase also found in some diseases may, at least in part, be due to changes in the enzyme environment rather than an actual increase in enzyme protein.

SECTION 9 GENERAL DISCUSSION AND CONCLUSIONS

The work presented in this thesis gives further support to the concept that the metabolism of xenobiotics may in some cases be an activating or toxication process.

4-Aminobiphenyl and 4-acetamidobiphenyl were shown to be converted to N- and ring hydroxylated derivatives (Section 2), some of which were demonstrated to be more carcinogenic than the parent compound (Section 6 and references cited therein).

Unfortunately the N-acetylated compounds were not all available at the time to allow them to be tested using the mouse neonate technique or to examine their effects on lysosomal membrane stability (Section 7). It is clear that aromatic amine acetylation and the deacetylation of arylacetamido compounds are metabolic pathways which have to be considered togetherwhen studying the metabolism of aromatic amines.

The evidence presented in Sections 4 and 5 shows that these reactions are far more specific than is perhaps generally thought. As the site of ring hydroxylation is to some extent governed by whether the amine function is free or acetylated, these conjugative and hydrolytic processes may play a role in the ultimate toxicity of an aromatic amine.

Regarding the nature of the toxic agent derived from 4-aminobiphenyl or 4-acetamidobiphenyl, no absolute correlation could be found between site of hydroxylation and species susceptibility to amine carcinogenesis (Section 2).

The results indicate that 4-aminobiphenyl is converted into two known toxicants viz. 4-hydroxylaminobiphenyl and 4-amino-3-hydroxybiphenyl, whilst 4-acetamidobiphenyl is converted into the corresponding toxic hydroxamic acid, as well as to ring hydroxylated metabolites presently thought to be innocuous.

As the parent compounds tend to produce their carcinogenic effect at different sites, - i.e. 4-aminobiphenyl usually produces urinary tract tumours whereas 4-acetamidobiphenyl produces hepatic tumours (see Section 1), - there does not seem any a priori reason to assume the requirement for a common intermediate toxicant, despite the similarity of their chemical structure.

In order for a toxicant to produce a deleterious effect on a biological system there has to be an interaction between the toxic stimulus and a biological receptor.

The interaction can be a direct reaction, i.e. one leading to the production of the ultimate biochemical lesion, or an indirect reaction, for example one which allows the release of an enzyme from an organelle or upsets the oxido-reduction status quo within the cell. In these reactions the formation of a stable covalent bond between toxicant and receptor is not a prerequisite as formation of an easily dissociable enzyme-inhibitor complex could product similar results. This concept has been amplified elsewhere (Gorrod, 1979).

In the case of carcinogenic substances, the insult which the susceptible cell receives has to be of a very special type: that is it has to be sufficiently great that the cellular repair mechanisms are unable to restore the cell to normality and yet sufficiently mild that cell death is not produced. It may be that the majority of cells attacked by a potential carcinogen do not receive such an insult and the tumour arises via the few (or even one) cells where just the correct conditions obtain.

It is worth considering the nature of the site of subcellular damage required in order to allow tumour development. Tumours are, using the most simplistic

definition, cell masses characterised by a lack of coordinated differentiation and a rather unrestrained growth pattern. Therefore one has to consider with which biological receptor the carcinogen would have to react in order to produce this effect. Cell growth, as exemplified by protein synthesis, functions under control mechanisms which have the ability to either allow more of a specific protein to be produced or to virtually "close down" its production. The exact mechanism(s) by which these cellular controls are mediated are not fully understood and are outside the scope of this section. Suffice it to say that interference with these mechanisms could account for the unrestrained cell growth (Pitot & Cho, 1965), and if these controls utilised proteins or peptides as repressor substances then a reaction between one of these substances and an aromatic amine or a hydroxylated metabolite could easily be envisaged. Indeed the reaction between 2-amino-1-naphthol and bovine serum albumin was reported by Nagasawa & Gutmann (1959) and confirmed by Belman & Troll (1962), although the latter authors questioned the nature of the moiety attached to the protein.

Bovine serum albumin is not typical of many of the proteins present in mammalian cells due to its high proportion of lysine (the amino acid where reaction with 2-amino-1-naphthol takes place). However, this protein is similar in many respects to the basic nucleo-proteins, the histones (Johns, 1971), and as histones interact with DNA it can be seen that any reaction which interferes with this interaction is likely to interfere with transcription of genetic information.

In view of the reaction of aminonaphthols with the ε-amino group of lysine cited earlier, it would not be surprising if these carcinogens reacted with other polyamines. Polyamines may very well play an important role in control of nucleic acid synthesis (Stevens, 1970) and have been implicated in the teratogenic effect of thalidomide (Fabro, Schumacher, Smith, Stagg & Williams, 1965). If by the reaction of the carcinogen with the histone or polyamine it caused dissociation of the nucleohistone complex the nucleic acid could very well become vulnerable to the degradative action of nucleases.

Further evidence that aromatic amines or their metabolites could react with nucleophilic centres in proteins or peptides came with the report by Boyland, Manson and Nery (1963) that aniline and 2-naphthylamine were excreted as mercapturic acids. It would seem that these metabolites are formed by reaction of the metabolically formed arylhydroxylamine with glutathione, followed by the known degradative pathway of glutathione conjugates.

An alternative mechanism involving a quinone-imine intermediate has been proposed (Boyland, Manson & Nery, 1963). In view of the fact that this reaction was observed with the non-carcinogenic amine, aniline, as well as with the carcinogenic 2-naphthylamine, it would seem unlikely that this reaction per se is involved in carcinogenesis, but it indicates that aromatic amine metabolites can react with nucleophilic centres.

In a further study using ^{14}C-labelled compounds, the binding of aniline and 2-naphthylamine to cellular proteins in vivo was compared (Roberts & Warwick, 1966).

No evidence was produced to suggest that protein binding was related to carcinogenesis as both amines were bound to about the same extent, 2-naphthyl-

amine being more highly bound to liver proteins than aniline, whereas aniline was bound to a great extent to kidney proteins.

More recently, Manson (1972) has shown that 2-amino-1-naphthylsulphate, 2-amino-1-naphthylglucosiduronic acid and bis(2-amino-1-naphthyl)phosphate have the ability to arylate N-acetylcysteine and glutathione in vitro, to give the corresponding S-(2-amino-1-naphthyl) derivatives. It is interesting that the related N-acetyl or N-formyl compounds were inactive as was 4-amino-3-biphenylyl sulphate, showing that this was not a general reaction of ortho-amino phenyl sulphates.

It may be that the controlling repressor or activator molecules are smaller molecules and Szent-Györgyi, Egyud and McLaughlin (1967) have suggested that certain ketones and aldehydes may have this function. If this is the case then certainly amines and their hydroxylated metabolites could react with them to form Schiff's bases and related compounds (Boyland & Nery, 1963) but it is difficult to see any specificity of reaction occurring.

Further, whilst such a reaction with a regulator may easily account for altered cell growth characteristics, it is unlikely that these would be passed on to daughter cells unless either residual amounts of carcinogen are also transferred or the genetic information of the cell is malfunctioning.

In order to produce a cell line with permanently altered genetic characteristics it is not unreasonable to propose that an alteration has to be introduced into the nuclear DNA. This alteration could involve producing a change in the base composition by a reaction of the carcinogen with a base leading to mismatching of complementary base pairs, or binding followed by excission of the affected area.

If reaction with nucleic acid is a prerequisite for carcinogenesis it might be expected that binding to target tissue DNA would be greater with carcinogens than with structurally similar inactive compounds. In fact most carcinogens examined do bind to nucleic acids and to proteins (Miller & Miller, 1966), and whilst DNA binding appears greater in susceptible organs in sensitive species compared to resistant species (Gutmann, Malejka-Giganti, Barry Rydell, 1972) few experiments have been performed to compare the binding of non-carcinogenic isomers. Roberts and Warwick (1966) did compare the binding of 2-naphthylamine and aniline residues to DNA after feeding the amines to rats, but did not show any difference between the carcinogenic and non-carcinogenc amine. The binding of 2-naphthylamine to cellular constituents was reinvestigated in mice of two strains by Hughes and Pilczyk (1969) who showed that the liver of the susceptible strain (CBA) bound 2-naphthylamine to all cellular macromolecules to a greater extent than did the non-sensitive C_{57} strain.

Following the discovery that 2-acetamidofluorene was excreted as the N-hydroxylated metabolite (Cramer, Miller & Miller, 1960) it was soon realised that this metabolite was unique in that, unlike the ring hydroxylated compounds, it possessed enhanced carcinogenic activity compared to the parent (Miller, Miller & Hartmann, 1961). This metabolite, the excretion of which showed a good correlation with carcinogenic activity (Table 9.1), also produced a much higher level of binding to macromolecules when fed to experimental animals (Willard & Irving, 1964; Marroquin & Farber, 1965; Miller, Cooke, Lotlikar & Miller, 1964), evidence suggesting that N-hydroxylation gave the reactive form derived from the amide. This compound, however, was disappointingly unreactive towards protein or nucleic acids when examined in vitro.

Table 9.1 The relationship between N-hydroxylation of 2-acetamidofluorene and carcinogenicity in various species

SPECIES	CARCINOGENIC EFFECT of 2 A A F	% DOSE EXCRETED AS N-HYDROXY DERIVATIVE
RABBIT	+	13 - 20
RAT	+	0.3 - 15
DOG	+	5.2
HAMSTER	+	5.0
MOUSE	+	1.8 - 2.3
CAT	+	1.5
STEPPE-LEMMING	-	TRACE
GUINEA-PIG	-	0
RAINBOW-TROUT	-	0

constructed from Weisburger and Weisburger (1973)

A similar lack of reactivity with nucleic acids by 2-naphthylhydroxylamine in vitro had been observed by Troll, Belman & Levine (1963) and Krick (1965); this latter author did, however, find a reaction between both 2-hydroxylamino-fluorene and 4-hydroxylaminobiphenyl and DNA at acid pH, and suggested that binding occurred via the C-8 position of guanine.

During in vitro experiments using the acetic acid ester of N-hydroxy-2-acetamidofluorene, Miller, Juhl and Miller (1966) observed a surprising reactivity with guanine present in DNA and RNA; this observation initiated the development of the concept that a second activating step was required in order to produce the ultimate carcinogenic agent.

Support for this idea gained strength when Lotlikar, Scribner, Miller and Miller (1966) showed that N-acetoxy-2-acetamidofluorene and N-benzoyloxy-N-methyl-4-aminoazobenzene reacted with methionine, whether free or as a peptide or when incorporated into protein. These reactions occurred under mild conditions where reaction with the N-hydroxy compound could not be detected. Treatment of the reaction mixtures with alkali produced the corresponding ortho-S-methyl compound and homoserine lactone. The proposed reaction mechanism is shown in Figure 9.1.

This mechanism raises two important points to consider, viz. the nature of the biological esterifying group and the determinant receptor molecule.

The first question has been studied in three ways: one by manipulating biochemical in vitro systems to allow the production of esters from various cofactors acting as biological acylating agents; a second by determining the carcinogenic or mutagenic activity of compounds under conditions

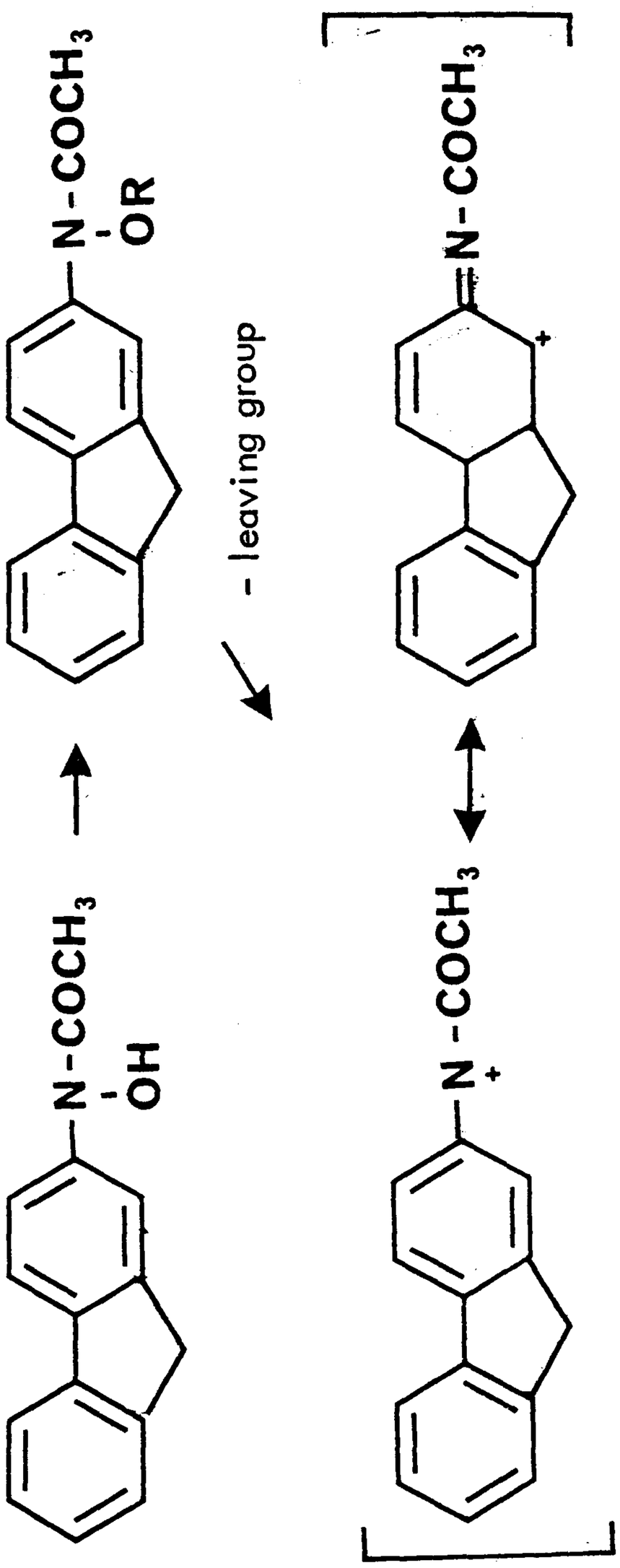


Fig. 9.1 The reaction of conjugates of N-hydroxy-2-acetamidofluorene with methionine

whereby one route of esterification is likely to be altered; and lastly by the chemical synthesis of potential conjugates of carcinogenic hydroxamic acids.

The glucosiduronate conjugate was the first ester of a xenobiotic hydroxamic acid to be discovered (Cramer, Miller & Miller, 1960) so that this ester was a likely candidate as the reactive ester. However, difficulties in obtaining reasonable quantities of this compound meant that considerable work was carried out using the acetoxy compound referred to earlier. This type of compound had not been found in vivo, and Lotlikar and Luha (1971a) investigated the possible formation of acetyl esters in biological systems.

Surprisingly, these authors found that reaction of hydroxamic acids with acetyl CoA occurred spontaneously. This reaction, which has a pH optimum between 10 and 11, was greatly influenced by the nature of the buffer used. No evidence was produced to show that this reaction was enhanced by the presence of tissue preparations, so that the formation of acetyl esters of hydroxamic esters in vivo, whilst being conceivable, has not yet been demonstrated.

Similar results were obtained using S-adenosylmethionine (Lotlikar, 1968), inorganic phosphate (Lotlikar & Wasserman, 1970), adenosine triphosphate (King & Phillips, 1968) and carbamyl phosphate (Lotlikar & Luha, 1971). In all cases, except methylation, a reactive ester is formed which has the ability to react with nucleophilic centres in nucleic acids or proteins and with methionine.

Another ester of hydroxamic acids can be formed by the transfer of the sulphate moiety from phosphoadenosine phosphosulphate under the influence of a sulpho-transferase (King & Phillips, 1968; De Baun, Rowley, Miller & Miller , 1968). The proposed structures of these esters are shown in Figure 9.2.

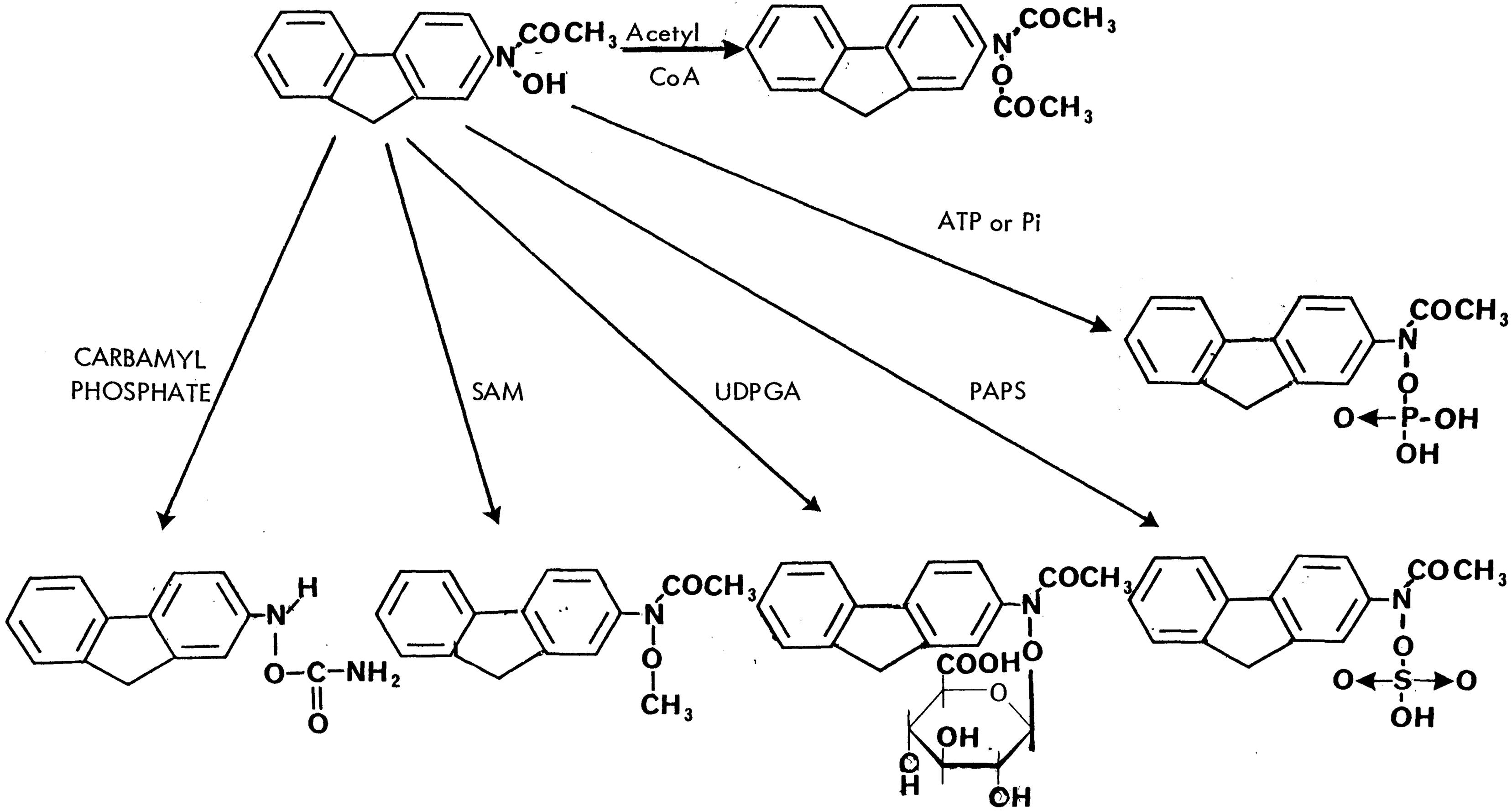


Fig 9.2 Potential metabolic routes to the formation of more reactive compounds from arylhydroxamic acids

Whilst considerable evidence has accumulated to support the idea that a reactive ester has to be formed, this is not universally accepted and other routes to a reactive molecule have been proposed. These are shown in Figure 9.3. Forrester, Ogily and Thomson (1970) and Bartsch, Traut and Hecker (1971) showed that N-hydroxy-2-acetamidofluorene was converted into a nitroxide radical under the influence of certain one-electron oxidants. The nitroxide radical undergoes a disproportionation reaction to produce N-acetoxy-2-acetamidofluorene and 2-nitrosofluorene, both of which are carcinogenic. This reaction can be carried out enzymically under the influence of peroxidase in the presence of hydrogen peroxide. (Figure 9.3,route A. Bartsch & Hecker, 1971; Floyd & Soong, 1977). Whilst this reaction undoubtedly produces reactive compounds, as measured by binding with nucleic acids, the nitroxide radical has been shown not to be an obligatory intermediate as neither cyanide or sulphite inhibit the peroxidase-induced binding to nucleic acids in vitro (King, Bednar & Linsmaier-Bednar, 1973). It was shown by Booth (1966) that hydroxamic acids could act as acetyl donors to a variety of amine acceptors and this observation was extended by Bartsch, Dworkin, Miller and Miller (1972, 1973) who proposed that aryl-hydroxylamines could also act as acetyl acceptors from hydroxamic acids to form O-acetyl derivatives which are reactive towards nucleophilic centres (Fig.9.3, route B). In order to get a suitable acceptor it would be necessary to hydrolyse one molecule of the parent hydroxamic acid (Fig.9.3, route C); this process is known to readily occur in tissue preparations (Irving, 1966).

An altemative proposal was put forward by King (1974) who showed that an enzyme was present in animal tissues which could transfer the acetyl group from the nitrogen to the oxygen of hydroxamic acids, thereby producing a reactive

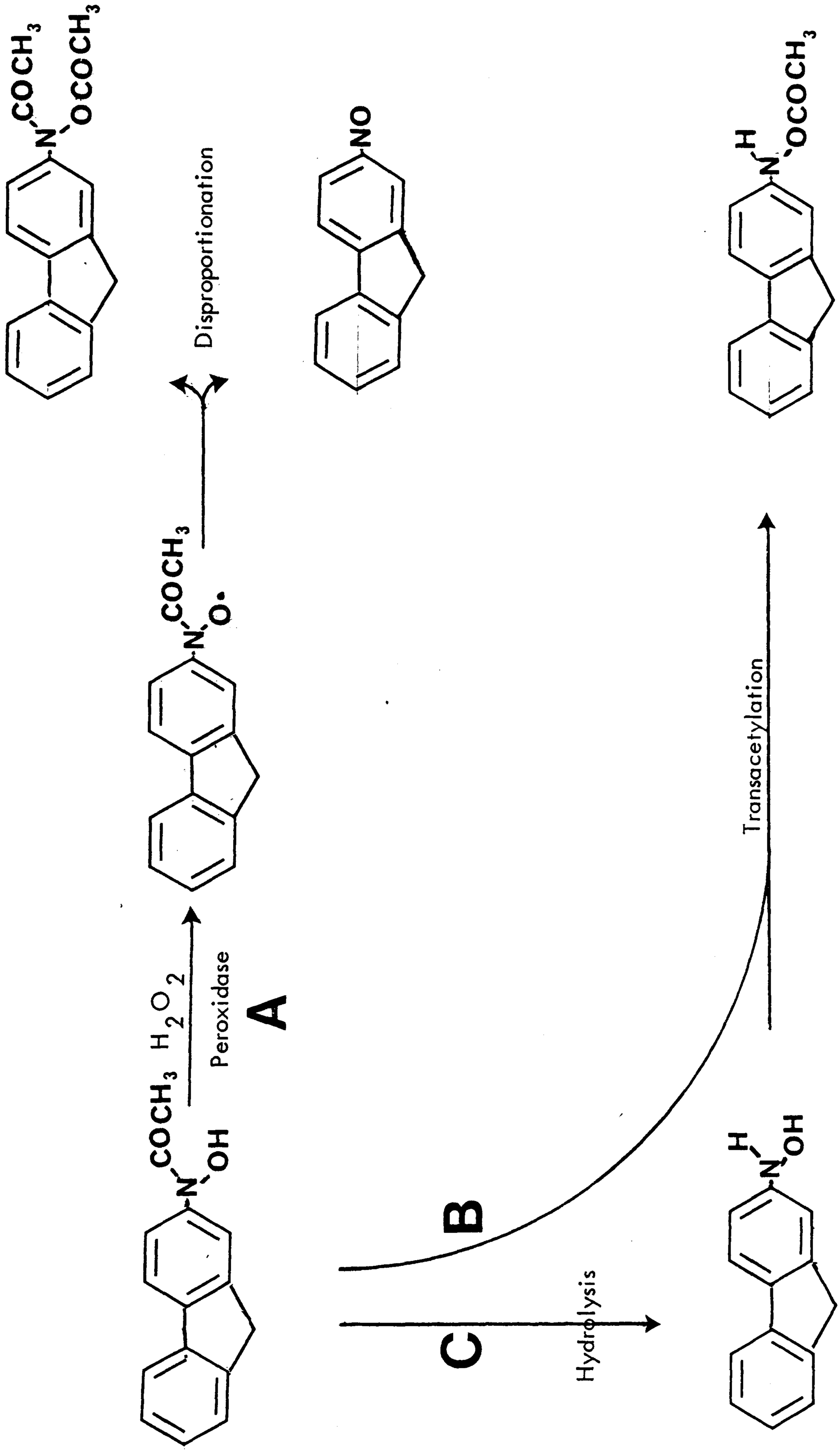


Fig. 9.3 Alternative processes proposed for the generation of reactive species

acetoxyamine (Fig.9.3 route B). Therefore, it can be seen that several processes are available which can produce a reactive species; of course the question as to which is the important route has been asked by many workers, yet the evidence is not unequivocal.

As described earlier, King & Phillips (1968) and DeBaun et al. (1968) proposed that the sulphuric acid esters of hydroxamic acids were involved in carcinogenesis. Support for this idea came from additional sources. The first was data gained from carcinogenicity tests on animals receiving acetanilide as well as either 2-acetamidofluorene or N-hydroxy-2-acetamidofluorene (Weisburger, Yamamoto, Williams, Grantham, Matsushima & Weisburger, 1972) where it was shown that acetanilide inhibited tumour formation by acetamidofluorene, possibly by inhibiting N-hydroxylation (Grantham, Mohan, Yamamoto, Weisburger & Weisburger, 1968). The carcinogenicity of acetamidofluorene was not restored by dietary sodium sulphate supplementation. A similar inhibition of tumour formation was produced by acetanilide when N-hydroxy-2-acetamidofluorene was administered to rats; in this case the inhibition could be partially reversed by additional dietary sulphate (Table 9.2.). This data was consistent with the idea that p-hydroxyacetanilide, formed as the major metabolite of acetanilide, utilised the available sulphate in forming a conjugate (Buch, Rummel, Pfleger, Eschrich & Texter, 1968), thereby preventing or minimising the formation of the sulphate conjugate of N-hydroxy-2-acetamido-fluorene and aarcinogenicity. Dietary sulphate was thought to restore the the sulphate conjugation of the hydroxamic acid and carcinogenicity.

Table 9.2 The influence of dietary acetanilide and inorganic sulphate on tumorigenesis by 2-acetamidofluorene and N-hydroxy-2-acetamidofluorene

	Diet		% Animals with liver neoplasms
1	2-acetamidofluorene	0.03%	100
2	2-acetamidofluorene plus acetanilide	0.03% 0.8%	10
3	2-acetamidofluorene plus acetanilide plus sodium sulphate	0.03% 0.8% 0.84%	0
4	N-hydroxy-2-acetamidofluorene	0.032%	100
5	N-hydroxy-2-acetamidofluorene plus acetanilide	0.032% 0.8%	10
6	N-hydroxy-2-acetamidofluorene plus acetanilide plus sodium sulphate	0.032% 0.8% 0.84%	60

Date from Weisburger et al. (1972)

Unfortunately, when the experiments were repeated using p-hydroxy-acetanilide in addition to the N-hydroxylated carcinogen only a weak inhibition of tumour formation was observed compared to the effect with acetanilide, and carcinogenicity was not restored by additional dietary sulphate (Yamamoto, Williams, Richardson, Weisburger & Weisburger, 1973). Later work by Mohan, Grantham, Weisburger, Weisberger, and Idoine (1976) showed that p-hydroxyacetanilide increased hepaticglucuronyl transferase and suggested that shunting more of the N-hydroxy carcinogen through this pathway accounted for the protective effect and the failure of sulphate to restore carcinogenic activity.

A further anomaly is apparent in that whereas the levels of hydroxamic acid sulphotransferase showed a very good correlation with various physiological conditions and cancer induction (DeBaun, Rowley, Miller & Miller, 1968; De Baun, Miller & Miller, 1970), this enzyme did not appear to activate N-hydroxy-2-acetamidofluorene when examined in the Ames Salmonella test for mutagenicity (Moulder, Hinson, Nelson & Thorgeirsson, 1977; Andrews, Hinson & Gillette, 1978). This test usually gives a very high correlation with carcinogenic activity (McCann & Ames, 1976). Furthermore, whilst the sulphotransferase activity may correlate very well with hepatic tumour induction, this enzyme is absent in Zymbals gland and mammary glands (Irving & Veazey, 1971).

Neither was there any correlation observed between sulphotransferase activity and the activation of another group of N-oxygenated carcinogens derived by chemical N-oxidation of purines (McDonald, Stohrer & Brown, 1973).

King (1974) and King & Olive (1975) studied the acyltransferase referred to earlier and showed that the distribution of this enzyme in species, strains and tissues corresponded well with tumour formation from 2-acetamidofluorene. These authors also proposed that inhibition of this enzyme by acetanilide and p-hydroxyacetanilide could account for the inhibition of carcinogenesis observed with these compounds. Surprisingly no similar study on the distribution of the enzyme catalysing the biosynthesis of glucosiduronic acid conjugates of hydroxamic acids has been published, although their reactivity with nucleic acids etc. is well documented.

Following the isolation of the O-glucuronide of N-hydroxy-2-acetamido-fluorene by Hill and Irving (1967), this compound was found to react with RNA and DNA in vitro (Irving, Veazey & Russell, 1969; Irving, Veazey & Hill, 1969). The latter authors also found that the glucuronide bound more extensively to RNA and DNA when given to rats in vivo than did 2-acetamidofluorene, although less than the hydroxylated amide. Obviously if this was the only route to a reactive species more binding would have been expected; nevertheless it was concluded that the glucuronide ester could account for some of the binding to nucleic acids observed during carcinogenesis by the parent amide. Despite the reactivity of this glucuronide, both in vivo and in vitro, this substance and its triacetyl methyl ester were not carcinogenic in rats under conditions where the hydroxamic acid was (Miller, Lotlikar, Miller, Butler, Irving & Hill, 1968).

An analogous glucuronide is formed from N-hydroxy-4-acetamidobiphenyl (Irving, Russell & Kriek, 1972) and is also reactive towards nucleic acids and guanosine; however, this reactivity is very low, occurring at only about 3-4% of that observed from the glucuronide of N-hydroxy-2-acetamidofluorene.

From the above it can be seen that the nature of the reactive ester, if indeed one is required, is still a matter for conjecture. In studies on the related amides, phenacetin and paracetamol, it again appears that N-hydroxylation can give rise to a more toxic species (Nery, 1971; Hinson & Mitchell, 1976).

In these cases, ester formation also enhances the reactivity with glutathione or protein (Hinson, Nelson & Mitchell, 1977; Mulder, Hinson & Gillette, 1977 & 1978; Calder & Creek, 1976), but the relative importance of the respective esters in vivo has not been established. It is of some interest that structurally similar hydroxamic acids had entirely different degrees of activation by ester formation, so that whilst N-hydroxy-phenacetin and N-hydroxy-2-acetamido-fluorene were greatly "activated" by esterification with sulphate, N-hydroxyacetanilide, N-hydroxy-p-chloroacetanilide and N-hydroxy-2-acetamidonaphthalene were hardly affected by this process.

More recently Andrews, Hinson and Gillette (1978) have shown that whereas sulphation of N-hydroxy-2-acetamidofluorene decreased mutagenesis towards Salmonella TA1538, a soluble enzyme present in rat liver greatly enhanced the mutagenic activity. These authors suggest that the enzyme hydrolyses the hydroxamic acid to the hydroxylamine which is known to be a potent frameshift mutagen. Certainly there is some evidence that an acetyl group is not always retained in the carcinogen moiety bound to nucleic acids (see later) and this approach would lead to a unifying concept of the mechanism of tumour initiation of amines and amides.

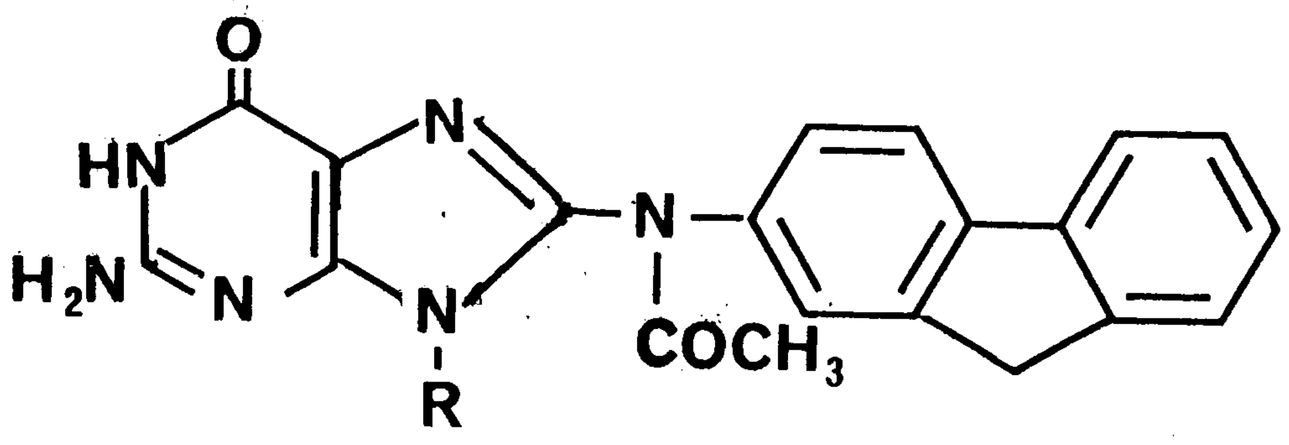
In the case of the dog any mechanism proposed could not involve an acetyl group, as this species lacks the ability to acetylate aromatic amines (see Sections 2, 3 and 4). As described in Section 2, evidence has accumulated to suggest that

excretion of N-hydroxy-4-aminobiphenyl occurs in this species and is related to bladder tumour formation; however, the instability of this compound suggested that it was unlikely to have survived synthesis in the liver, transport via the blood, excretion by the kidney and reabsortion from urine, unless it was present as a conjugate (Radomski, Roy & Brill, 1973). In 1977 Radomski, Heam, Radomski, Moreno and Scott succeeded in isolating a glucuronide conjugate of 4-hydroxylaminobiphenyl from dogs fed 4-aminobiphenyl. This compound, which was probably the N-glucuronide (Moreno & Radomski, 1978), was an active mutagen in several Salmonella tester strains, although possessing a lower activity than the corresponding hydroxylamine or nitroso compounds. It would be interesting to know if the corresponding O-glucuronide, which the authors claimed to have synthesised, was an active mutagen, as this would have a structure similar to the esters derived from hydroxamic acids discussed earlier.

Kadlubar, Miller and Miller (1977) have also considered the possibility of aryl hydroxylamines being excreted as glucuronic acid conjugates and succeeded in preparing the N-glucuronides of the N-hydroxy derivatives of 1 & 2-naphthylamine, 4-aminobiphenyl and 2-aminofluorene, These reactions were carried out in vitro using hepatic microsomal preparations from dogs, rats or humans.

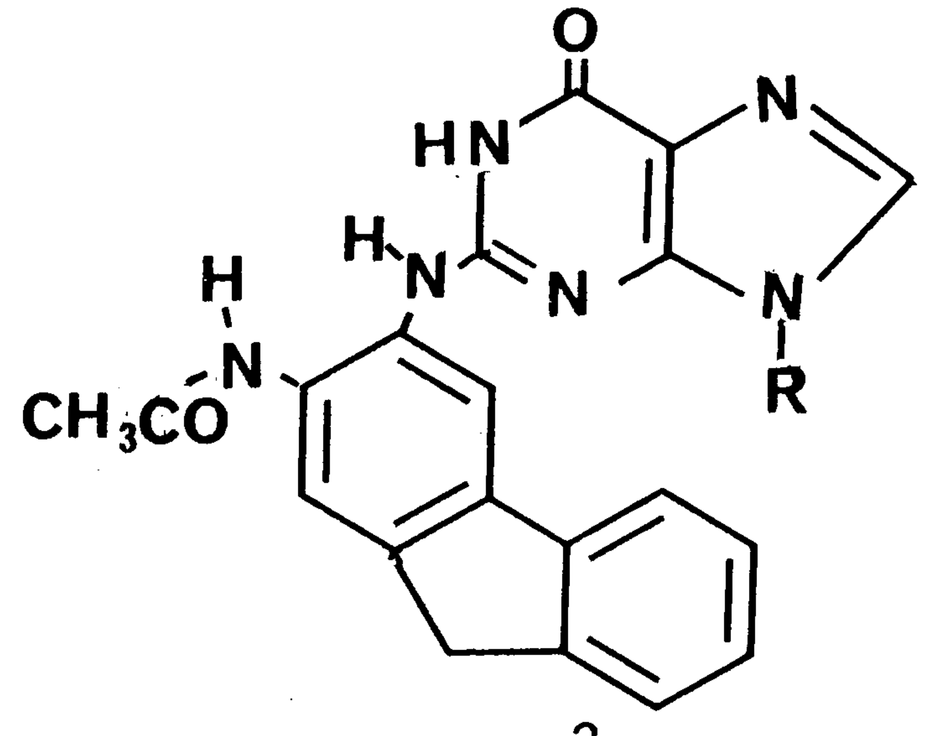
The finding that hydroxylamines (Kriek, 1965) and esters of hydroxamic acids (Miller, Juhl & Miller, 1966 and references cited earlier in this Section) could react with nucleic acids, poses the question of the nature of the bound material and the base to which it is bound. Kriek (1965) and Miller, Juhl and Miller (1965) presented evidence that the base guanine was the principal site of the reaction in vitro. Many carcinogens have been found to react with DNA (Irving, 1973; Brookes, 1977) but few studies have elucidated the

nature of the bound material during tumorigenesis: i.e. in the susceptible tissue. Such a study has been carried out by Kriek and co-workers (Kriek, 1969, 1971, 1972, 1974; Westra, Kriek & Hittenhausen, 1976). The results from these elegant studies are summarised in Figure 9.4. It appears that attack on the C-9 position of guanine is the major route to the formation of bound material in either DNA or RNA. This material may be present either in an acetylated or deacetylated form. This material seems to have a relatively short half life of seven days (in DNA) and is probably cleared from the nucleic acid by a repair mechanism. An analogous reaction product has been observed for 4-acetamidobiphenyl (Kriek, 1971), although occurring at much lower levels than with 2-acetamidofluorene. During their work with 2-acetamidofluorene a further component was found which wasbound only to DNA; this material, which accounted for about 20% of the initial bound material, had a much longer half life and could easily be detected even eight weeks after the carcinogen had been given to rats. The structure of this material has been elucidated and it has been shown to arise via attack on the amino group of guanine (Fig.9.4) to give 3-deoxyguanosin-N^2-yl)-2-acetamidofluorene. It is clear that this type of persistent bound material can seriously affect the function of cellular DNA; thus it would be unlikely to successfully base pair with messenger RNA and would thereby affect protein synthesis, perhaps leading to deletion of a specific protein as was suggested many years ago to occur in carcinogenesis (Miller & Miller, 1947; cf. Potter, 1964). This binding could also account for alterations in Tm, buoyant density and RNA polymerase priming activity previously observed with carcinogen-treated DNA (Troll, Rinde Day, 1969) and the loss of transforming activity (Maher, Miller, Miller & Szybalski, 1968; Maher, Miller, Miller & Summers, 1970).

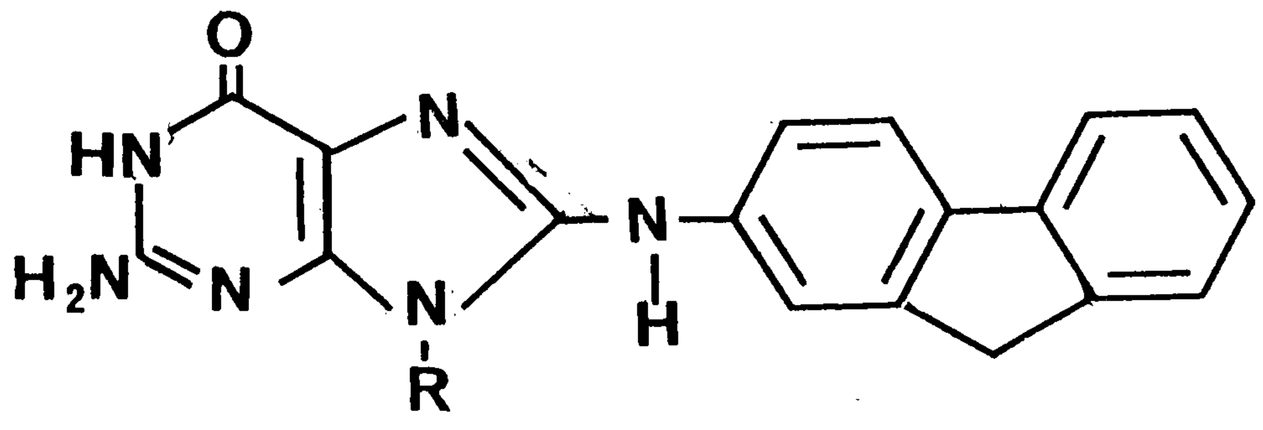


N-(guanosin-8-yl)-2-AAF
accounts for 75-80% of carcinogen bound to rRNA

N-(deoxyguanosin-8-yl)-2AAF
accounts for 10% of carcinogen bound to DNA



3-(deoxyguanosin-N^2-yl)-2-AAF
accounts for 20% of carcinogen bound to DNA



N-(guanosin-8-yl)-2-AF
accounts for 20-25% of carcinogen bound to rRNA

N-(deoxyguanosin-8-yl)-2-AF
accounts for 70% of carcinogen bound to DNA

R = ribose in RNA

R = deoxyribose in DNA

Fig. 9.4 Structures of the bound forms of 2-acetamidofluorene found in nucleic acids (constructed from Kriek, 1974)

It is interesting that the N-glucuronide of N-hydroxy-1-naphthylamine also reacts with nucleic acids in vitro (Kadlubar, Miller & Miller, 1978). In this case the base attacked is still principally guanine, but binding occurs via the O^6 position of the base and either the N-or 2-position of the carcinogen to give either N-(deoxyguanosin $-O^6$-yl)-1-naphthylamine or 2-(deoxyguanosin $-O^6$-yl)-1-naphthylamine.

In studies using the related compound N-hydroxy-2-acetamidophenanthrene, Scribner and Naimy (1973, 1975) showed that in this case binding to nucleic acids occurred via attack on adenine as well as guanine, so that it is surprising that in the case of 2-acetamidofluorene binding appears to occur exclusively via guanine. It may be that binding to other bases does occur but at such a low level that they are not detectable by contemporary methods. Scribner and Naimy (1975) have developed an interesting concept to account for differences in reactivity observed between different N-acetoxy-acetamido compounds and purine bases. Firstly, they propose that the rate of reaction depends upon whether a compound hydrolyses to an N-hydroxyacetamido compound or to a N-acetyl-N-arylnitrenium ion, which they propose as the reactive species. Secondly, they claim that the site of reaction can be predicted from molecular orbital calculations carried out on the various nitrenium ions. High reactivity of the nitrogen leads to reaction via carbon C-8 of guanine; high reactivity of carbon leads to reaction via an amino group in either adenine or guanine. Further work using a range of compounds will be needed to explore these ideas.

It is perhaps unfortunate, from a unifying point of view, that this "persistent" material still contains an acetyl function, as such a situation cannot obtain with aromatic amines in the dog, which nevertheless is susceptible to aromatic amine-

induced tumours. It may be that ortho-aminophenols such as 4-amino-3-hydroxy-biphenyl, are the active agents as discussed in Section 1.6; certainly they are carcinogenic when implanted into the bladders of mice and are formed at high levels in species susceptible to the carcinogenic effect of the parent amines. If this is the case, then an alternative method of binding with DNA and affecting genetic processes presents itself. It is known that 4-amino-3-hydroxybiphenyl is oxidised to 1,7-diphenyl-4-amino-3H-isophenoxazine-3-one (Nagasawa, Gutmann & Morgan, 1959; Fig.9.5), a substance which is carcinogenic in the mouse bladder implantation test (see references in Section 1.6). This substance is structurally related to the phenoxazone cinnabarinic acid, a compound formed by nuclear enzymes from 3-hydroxy-anthranilic acid (Subba-Rao, Jegannathan & Vaidyanathan, 1965),and to actinomycin D (Fig.9.5).

As actinomycin D binds very strongly with nucleic acids (Hamilton, Fuller & Reich, 1963) it may be that other isophenoxazones derived from carcinogenic ortho-aminophenols can react in the same way.

Kiese & Lenk (1971) observed the formation of a new compound in rabbit urine following the administration of 4-chloroaniline. This material was isolated after the urine had been treated with glucuronidase and sulphatase and was tentatively characterised as 3-amino-7-chlorophenoxazone-2, although by analogy with the product from 4-amino-3-hydroxybiphenyl the present author would have thought it more likely to be 1,7-dichloro-4-amino-3H -isophenoxazone, unless chlorine can be eliminated during the condensation reaction.

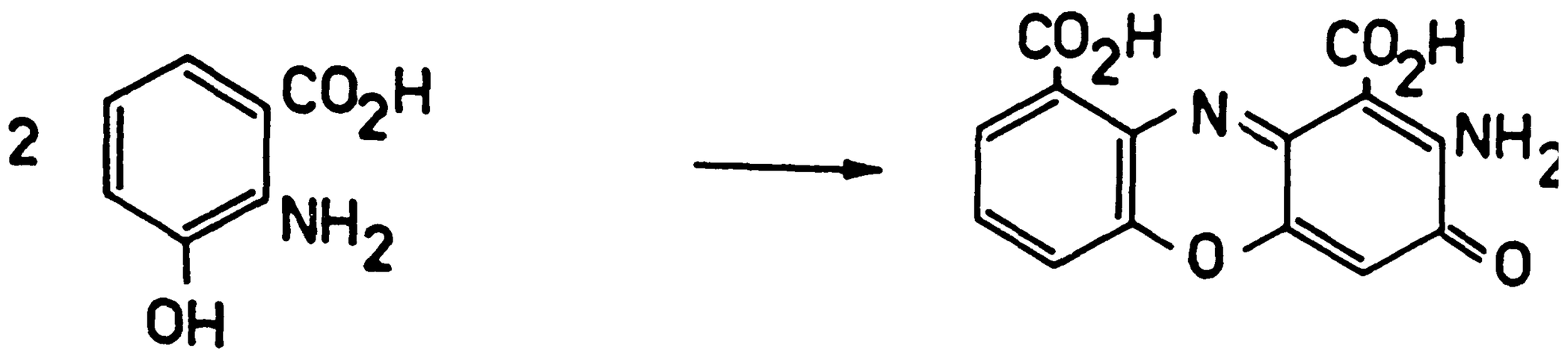
In respect to their similarity to actinomycin D it is interesting that 2-amino-1, 4-naphthoquinone, a compound which is formed by oxidation of the carcinogenic 2-amino-1-naphthol (Belman, Ferber & Troll, 1967), has been proposed as a model

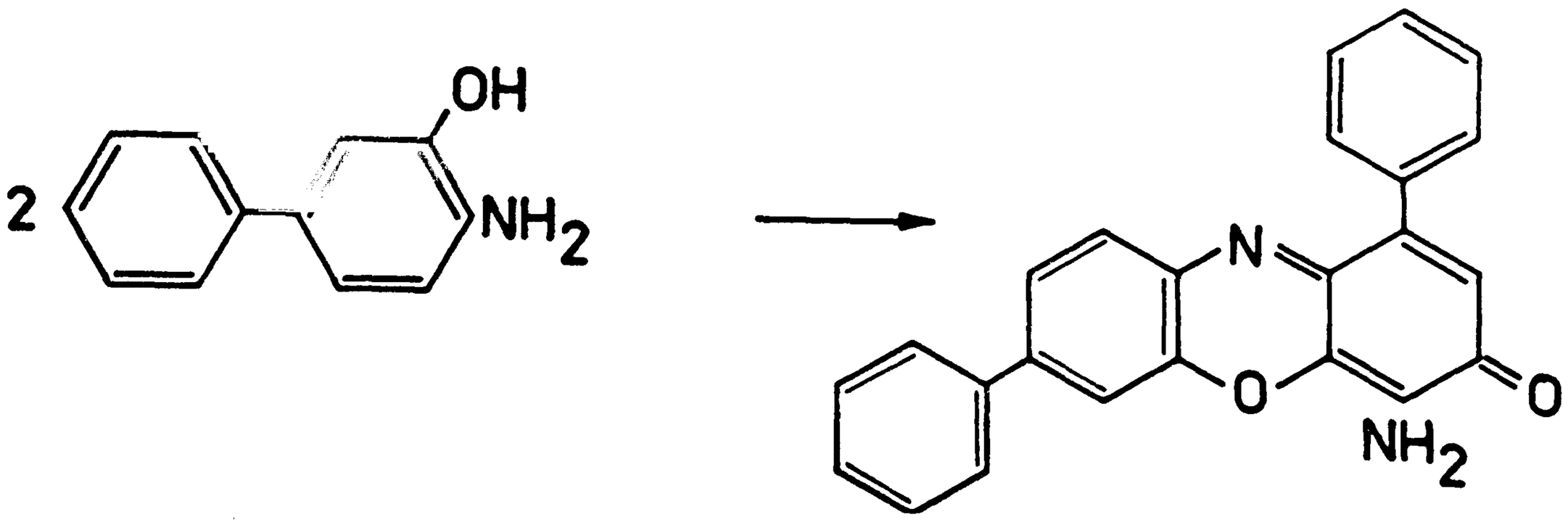
Table 9.4 The mutagenic activity of 4-aminobiphenyl and some derivatives

TEST STRAIN	Amount of Substance/plate (μg)	4-aminobiphenyl	4-hydroxylamino-biphenyl	N-hydroxy-4-acetamidobiphenyl	N-acetoxy-4-acetamidobipenyl
E. coli	500	31*	NE	79	107
WPZ uvr (P)	100	77	NE	95	152
(for detecting	50	103	471	95	107
Base-pair mutations)	10	NE	195	NE	NE
	5	NE	218	NE	NE
control	0	70	62	84	63
S.typhimurium	1000	33	NE	NE	NE
TA98	500	35	NE	41	283
(for detecting	100	55	NE	41	80
Frameshift mutations)	50	NE	540	41	62
	10	NE	338	NE	NE
	5	NE	198	NE	NE
control	0	53	32	34	36
S.typhimurium	1000	156	NE	NE	234
TA100	500	149	NE	181	190
(for detecting	100	163	NE	176	195
Base-pair mutations)	50	NE	307	198	115
	10	NE	257	NE	NE
	5	NE	206	NE	NE
	1	NE	143	NE	NE
control	0	191	134	179	155

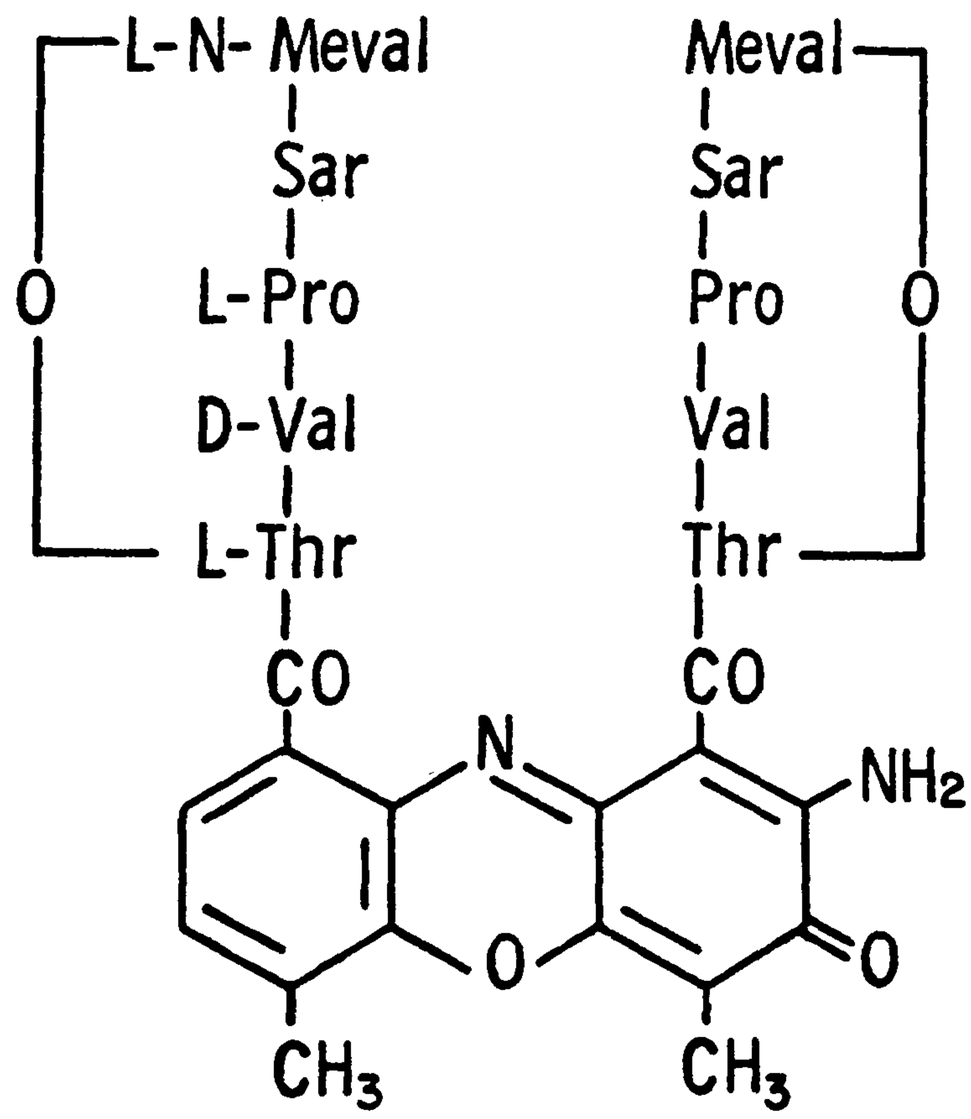
NE = Not examined

*figures are number of colonies per plate





Formation of isophenoxazones



Actinomycin D

Fig. 9.5 The oxidation of ortho-aminophenols to isophenoxazones and their structural relationship to Actinomycin D

compound for the study of actinomycins (Martin, 1964). A structural analogue derived from 2-amino-1-fluorenol was very strongly bound to proteins under conditions where N-hydroxy 2-acetamidofluorene was not (Gutmann & King, 1962).

It may be that the ideas discussed in this Section are pertinent not only to amine and amide carcinogenesis, but are also worth considering in relation to other areas of amine and amide toxicity. It has long been known that the N-hydroxy-derivatives of 1- and 2-naphthylamine are mutagenic to certain strains of E.coli (Perez & Radomski, 1965; Belman, Troll, Teebor & Mukai, 1968). Recently, because of the high correlation between mutagenicity and carcinogenicity obtained by Ames and co-workers (see McCann, Choi, Yamasaki & Ames, 1975), there has been a great interest in this area. The mutagenicity of 4-aminobiphenyl, 4-hydroxylaminobiphenyl, N-hydroxy-4-acetamidobiphenyl and N-acetoxy-4-acetamidobiphenyl in a number of bacterial test systems has been examined as part of a programme to assess the role of N-oxidation in mutagenesis caused by a variety of nitrogen-containing structures (Pai, Bloomfield, Jones & Gorrod, 1978).

The data presented in Table 9.4 is derived from some recent work by Miss Pai. In agreement with other workers she found that 4-aminobiphenyl was inactive as a mutagen in three test systems. These systems detect mutagens which act either by frameshift mutation or a base-pair substitution mechanism. In contrast the corresponding hydroxylamine was highly mutagenic and apparently could act by either mechanism. However, it may be that under the conditions of assay some of the hydroxylamine was oxidised to 4-nitrosobiphenyl, as Radomski et al. (1977) have shown that 4-nitrosobiphenyl is a potent frameshift

mutagen using TA98 as tester strain. 4-Nitrosobiphenyl and a number of other nitroso compounds have previously been shown to be powerful mutagens towards Salmonella typhimurium (Ames, Gurney, Miller & Bartsch, 1972).

The corresponding N-acetyl derivative was again inactive, suggesting that some activating process was lacking; however, when the synthetic acetoxy ester was examined this was found to be a mutagen, although only at high concentration compared to the hydroxylamine. These results, which were obtained without the addition of any mammalian hepatic enzyme, support the idea that in order for a hydroxamic acid to be active a further metabolic step is required. Our experiments using the acetoxy compound did not indicate greatly enhanced activity at low concentrations, whereas removal of the N-acetyl group certainly did. This suggests that the acyltransferase or a hydrolytic process as discussed earlier are required. In studies with N-hydroxy-2-acetamidofluorene support for the acyl-transferase (Weeks, Allaben, Louie, Lazear & King, 1978) and the hydrolase (Stoat, Baptist, Matney & Shaw, 1976; Andrews, Hinson & Gillette, 1978) mechanisms are available, and presently it is not possible to distinguish between these two hypotheses.

If deacetylation is a prerequisite for mutagenesis then the mechanism must lead to nucleic acid-bound products which are different from those found during carcinogenesis where the acetamido group remains intact.

In a series of experiments designed to examine the mutagenicity of derivatives of 2-acetamidofluorene, Fahmy and Fahmy (1972) used Drosophila melanogaster as the test organism. This study showed that the highest mutagenic activity was again associated with esters of the hydroxamic acid. Some differentiation of effect was observed; the N-acetoxy compound produced a wide range of mutational

effects, whereas the N-sulphate produced rather specific effects.

Nelson and Thorgeirsson (1972) examined the mutagenicity of a wide range of 2-acylaminofluorenes towards S.typhimurium. These authors concluded that the mutagenicity of these compounds correlated with the degree of N-hydroxylation undergone by the substrate.

The mechanism of the mutagenic action of hydroxylamines has recently been reviewed by Budowsky (1976).

The results of mutagenicity testing of compounds containing nitrogen, together with their N-oxidised derivatives and conjugates, are obviously as difficult to interpret as those obtained during carcinogenicity studies.

One's final conclusion has to be that despite the enormous amount of work which has been carried out since the recognition of amine-induced cancer in man and its reproduction in experimental animals, much still remains to be done in elucidating the mechanism of amine and amide carcinogenesis. Perhaps one of the most satisfying things to appear is the idea that many diverse structures may be activated via enzyme systems which had hitherto been considered as detoxifying systems (See Gorrod, 1979 and references cited therein).

These enzymes have the ability to metabolise many naturally-occurring substances, as well as xenobiotics, and as these enzymes vary from subject to subject they may be able to produce active carcinogens in one subject yet completely protect another. If this is true, then a knowledge of substrate specificity and route of metabolism may ultimately help to reduce the incidence of cancer caused by both endogenous and exogenous compounds.

In this case we may truly be "at the beginning of the end" rather than, as stated by the late Sir Alexander Haddow in his address to the International Cancer Congress in London (1958) "at the end of the beginning".

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ADDENDUM

The data contained in this addendum was obtained by Dr. M. R. Kibby, Department of Biochemistry, University of Strathclyde, Glasgow, Scotland using an ALGOL Hückel molecular orbital programme devised by Krüger-Theimer and Hansen (1966).

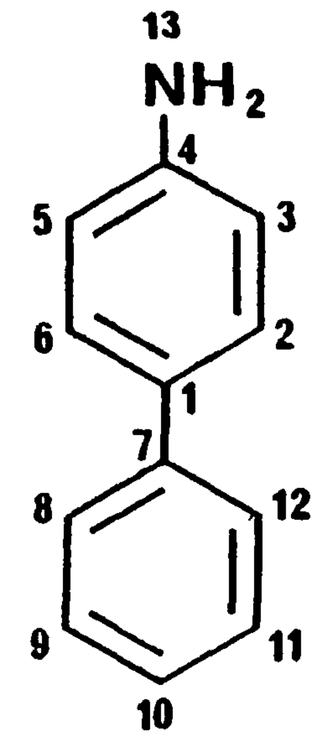
The numbering of the atoms within the molecules is in accordance with the diagrams shown and not the conventional system. This is necessary for the operation of the computer.

13/04/69 49/38/46

PROGRAM #SDPY HUECKEL MOLECULAR ORBITAL CALCULATION

4=AMINOBIPHENYL

RHO 1.0008 -6



4-Aminobiphenyl

NUMBER OF CALCULATED ATOMS 13 (WITHOUT SINGLE AND AMINO GROUP HYDROGEN)

ATOM NO.	ATOM	FORMAL ELECT.	COULOMB INT.	ELECTR. CHARGE	NET CHARGE	FREE VALENCE	SELF-ATOM POLARIz.	SUPER-DELOCAL.	ENERGY OF MOLECULAR ORBITAL	
1	C	1	1.00	1.054	-0.054	0.138	0.351	1.063	-2.315	
2	C	1	1.00	0.995	0.005	0.432	0.420	0.902	-2.026	
3	C	1	1.00	1.071	-0.071	0.436	0.418	1.146	-1.572	
4	C	1	1.00	0.942	0.058	0.123	0.349	0.816	-1.000	
5	C	1	1.00	1.071	-0.071	0.436	0.418	1.146	-1.000	
6	C	1	1.00	0.995	0.005	0.432	0.420	0.902	-1.000	
7	C	1	1.00	0.998	0.002	0.123	0.343	0.768	-0.460	HIGHEST FILLED ORBITAL
8	C	1	1.00	1.011	-0.011	0.439	0.426	0.980	0.770	LOWEST EMPTY ORBITAL
9	C	1	1.00	1.000	0.000	0.395	0.396	0.830	1.000	
10	C	1	1.00	1.010	-0.010	0.413	0.413	0.962	1.000	
11	C	1	1.00	1.000	0.000	0.395	0.396	0.830	1.386	
12	C	1	1.00	1.011	-0.011	0.439	0.426	0.980	1.928	
13	N	2	1.00	1.842	0.158	1.014	0.132	2.143	2.288	

DELENY 1.231

Molecular Orbital data for 4-aminobiphenyl

13/04/69 49/39/32

PROGRAM #SDPY HUECKEL MOLECULAR ORBITAL CALCULATION

4-ACETAMIDOBIPHENYL

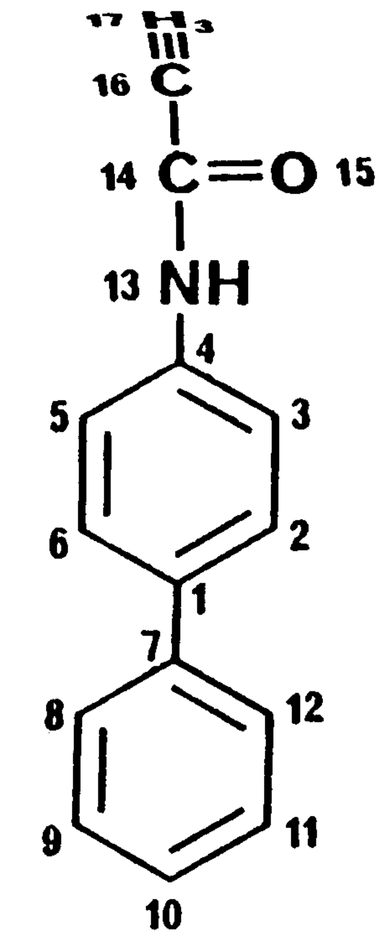
RHO 1.0008 -6

NUMBER OF CALCULATED ATOMS 17 (WITHOUT SINGLE AND AMINO GROUP HYDROGEN)

ATOM NO.	ATOM	FORMAL ELECT.	COULOMB INT.	ELECTR. CHARGE	NET CHARGE	FREE VALENCE	SELF-ATOM POLARIZ.	SUPER-DELOCAL.	ENERGY OF MOLECULAR ORBITAL	
1	C	1	0.00	1.045	-0.045	0.136	0.350	1.006	-2.964	
2	C	1	0.00	0.996	0.004	0.432	0.420	0.904	-2.302	
3	C	1	0.00	1.060	-0.060	0.431	0.416	1.085	-2.007	
4	C	1	0.00	0.949	0.051	0.138	0.355	0.826	-1.847	
5	C	1	0.00	1.060	-0.060	0.431	0.416	1.085	-1.553	
6	C	1	0.00	0.996	0.004	0.432	0.420	0.904	-1.009	
7	C	1	0.00	0.998	0.002	0.124	0.343	0.769	-1.000	
8	C	1	0.00	1.009	-0.009	0.438	0.426	0.966	-1.000	
9	C	1	0.00	1.000	0.000	0.395	0.396	0.830	-0.493	HIGHEST FILLED ORBITAL
10	C	1	0.00	1.009	-0.009	0.413	0.413	0.948	0.755	LOWEST EMPTY ORBITAL
11	C	1	0.00	1.000	0.000	0.395	0.396	0.830	1.000	
12	C	1	0.00	1.009	-0.009	0.438	0.426	0.966	1.000	
13	N	2	1.00	1.746	0.254	0.658	0.161	1.792	1.335	
14	C	1	-0.10	0.733	0.267	0.314	0.201	0.270	1.576	
15	O	1	1.20	1.409	-0.409	0.144	0.215	0.735	1.936	
16	C	1	0.00	1.052	-0.052	0.574	0.241	0.543	2.294	
17	H3	1	-0.20	0.930	0.070	-0.982	0.256	0.493	2.390	

DELENY 1.248

Molecular Orbital data for 4-acetamidobiphenyl



4-Acetamidobiphenyl

TOTAL PI-ENERGY IN GROUND STATE
14.000 ALPHA 18.747 BETA

DELOCALIZATION ENERGY 4.747 BETA

BONDED ATOM NOS.		FORMAL BONDS	RESONANCE INTEGRAL	BOND ORDER
1	2	1	1.00	0.609
1	6	2	1.00	0.609
1	7	1	1.00	0.376
2	3	2	1.00	0.692
3	4	1	1.00	0.605
4	5	2	1.00	0.605
5	6	1	1.00	0.692
7	8	2	1.00	0.616
7	12	1	1.00	0.616
8	9	1	1.00	0.677
9	10	2	1.00	0.659
10	11	1	1.00	0.659
11	12	2	1.00	0.677
4	13	1	0.90	0.400

4-Aminobiphenyl

49/38/59

TOTAL PI-ENERGY IN GROUND STATE
18.000 ALPHA 28.350 BETA

DELOCALIZATION ENERGY 5.169 BETA

BONDED ATOM NOS.		FORMAL BONDS	RESONANCE INTEGRAL	BOND ORDER
1	2	1	1.00	0.610
1	6	2	1.00	0.610
1	7	1	1.00	0.375
2	3	2	1.00	0.689
3	4	1	1.00	0.612
4	5	2	1.00	0.612
5	6	1	1.00	0.689
7	8	2	1.00	0.617
7	12	1	1.00	0.617
8	9	1	1.00	0.677
9	10	2	1.00	0.660
10	11	1	1.00	0.660
11	12	2	1.00	0.677
4	13	1	0.90	0.371
13	14	1	0.90	0.385
14	15	2	2.00	0.856
14	16	1	0.70	0.176
16	17	3	2.00	0.982

4-Acetamidobiphenyl

49/39/58

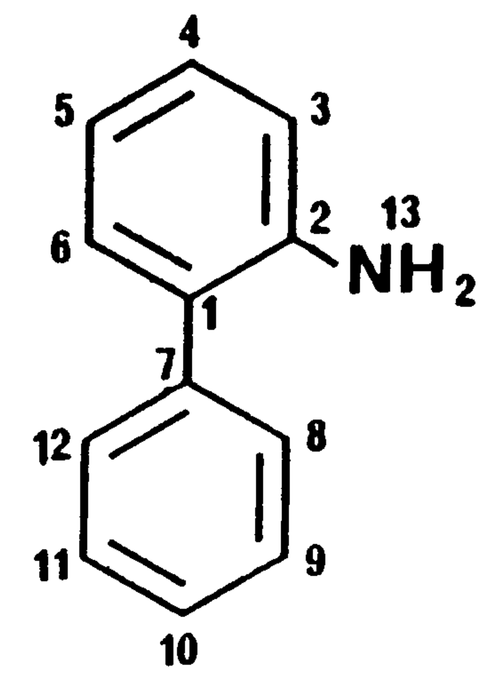
Bond Orders for 4-amino and 4-acetamidobiphenyl

13/04/69 49/38/29

PROGRAM #SDPY HUECKEL MOLECULAR ORBITAL CALCULATION

2-AMINOBIPHENYL

RHO 1.0008 -6



2-Aminobiphenyl

NUMBER OF CALCULATED ATOMS 13 (WITHOUT SINGLE AND AMINO GROUP HYDROGEN)

ATOM NO.	ATOM	FORMAL ELECT.	COULOMB INT.	ELECTR. CHARGE	NET CHARGE	FREE VALENCE	SELF-ATOM POLARIZ.	SUPER-DELOCAL.	ENERGY OF MOLECULAR ORBITAL	
1	C	1	1.00	1.065	-0.065	0.158	0.359	1.080	-2.354	
2	C	1	1.00	0.939	0.061	0.142	0.357	0.828	-1.965	
3	C	1	1.00	1.075	-0.075	0.439	0.420	1.153	-1.471	
4	C	1	1.00	0.995	0.005	0.407	0.407	0.886	-1.311	
5	C	1	1.00	1.057	-0.057	0.411	0.405	1.126	-1.000	
6	C	1	1.00	0.999	0.001	0.435	0.423	0.909	-0.803	
7	C	1	1.00	0.996	0.004	0.121	0.342	0.767	-0.472	HIGHEST FILLED ORBITAL
8	C	1	1.00	1.013	-0.013	0.440	0.427	0.982	0.735	LOWEST EMPTY ORBITAL
9	C	1	1.00	1.000	0.000	0.395	0.396	0.830	1.000	
10	C	1	1.00	1.011	-0.011	0.414	0.413	0.963	1.107	
11	C	1	1.00	1.000	0.000	0.395	0.396	0.830	1.319	
12	C	1	1.00	1.013	-0.013	0.440	0.427	0.932	1.913	
13	N	2	1.00	1.838	0.162	1.008	0.134	2.143	2.303	

DELENY 1.207

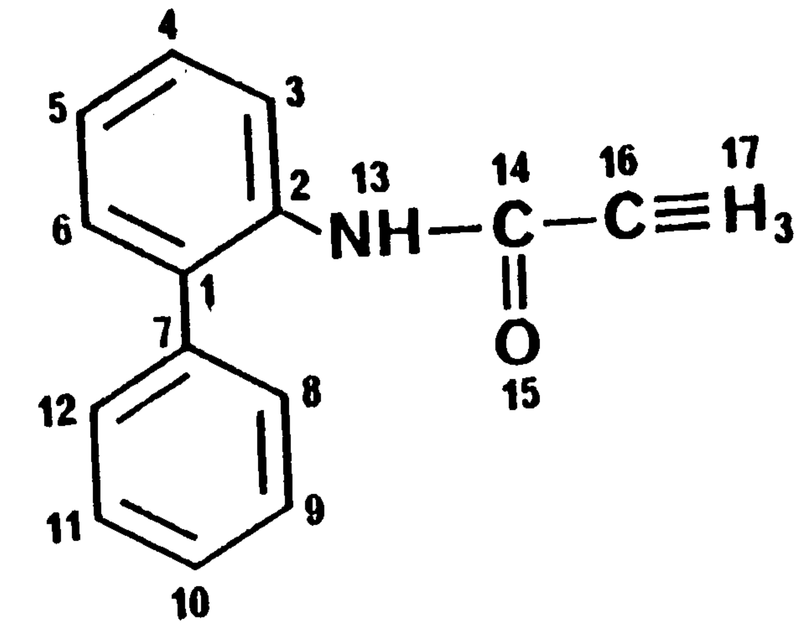
Molecular Orbital data for 2-aminobiphenyl

3/04/69 49/39/02

ROGRAM #SDPY HUECKEL MOLECULAR ORBITAL CALCULATION

-ACETAMIDOBIPHENYL

HO 1.00C& -6



2-Acetamidobiphenyl

NUMBER OF CALCULATED ATOMS 17 (WITHOUT SINGLE AND AMINO GROUP HYDROGEN)

ATOM NO.	ATOM	FORMAL ELECT.	COULOMB INT.	ELECTR. CHARGE	NET CHARGE	FREE VALENCE	SELF-ATOM POLARIZ.	SUPER-DELOCAL.	ENERGY OF MOLECULAR ORBITAL	
1	C	1	0.00	1.054	-0.054	0.154	0.358	1.020	-2.966	
2	C	1	0.00	0.946	0.054	0.157	0.364	0.838	-2.330	
3	C	1	0.00	1.063	-0.063	0.434	0.418	1.091	-1.958	
4	C	1	0.00	0.996	0.004	0.407	0.408	0.887	-1.848	
5	C	1	0.00	1.047	-0.047	0.408	0.404	1.068	-1.449	
6	C	1	0.00	0.999	0.001	0.435	0.423	0.909	-1.311	
7	C	1	0.00	0.997	0.003	0.121	0.342	0.788	-1.000	
8	C	1	0.00	1.011	-0.011	0.439	0.426	0.968	-0.809	
9	C	1	0.00	1.000	0.000	0.395	0.396	0.830	-0.507	HIGHEST FILLED ORBITAL
10	C	1	0.00	1.009	-0.009	0.413	0.413	0.950	0.729	LOWEST EMPTY ORBITAL
11	C	1	0.00	1.000	0.000	0.395	0.396	0.830	1.000	
12	C	1	0.00	1.011	-0.011	0.439	0.426	0.968	1.066	
13	N	2	1.00	1.743	0.257	0.653	0.162	1.792	1.318	
14	C	1	-0.10	0.733	0.267	0.315	0.201	0.270	1.559	
15	O	1	1.20	1.409	-0.409	0.143	0.215	0.735	1.917	
16	C	1	0.00	1.052	-0.052	0.574	0.241	0.543	2.290	
17	H3	1	-0.20	0.930	0.070	-0.982	0.256	0.493	2.398	

DELENV 1.236

Molecular Orbital data for 2-acetamidobiphenyl

NUMBER OF BONDS 14

TOTAL PI-ENERGY IN GROUND STATE
14.000 ALPHA 18.753 BETA

DELOCALIZATION ENERGY 4.753 BETA

BONDED ATOM NOS.		FORMAL BONDS	RESONANCE INTEGRAL	BOND ORDER
1	2	1	1.00	0.567
1	6	2	1.00	0.625
1	7	1	1.00	0.382
2	3	2	1.00	0.617
3	4	1	1.00	0.676
4	5	2	1.00	0.649
5	6	1	1.00	0.672
7	8	2	1.00	0.615
7	12	1	1.00	0.615
8	9	1	1.00	0.678
9	10	2	1.00	0.659
10	11	1	1.00	0.659
11	12	2	1.00	0.678
2	13	1	0.90	0.406

2-Aminobiphenyl

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NUMBER OF BONDS 18

TOTAL PI-ENERGY IN GROUND STATE
18.000 ALPHA 28.356 BETA

DELOCALIZATION ENERGY 5.174 BETA

BONDED ATOM NOS.		FORMAL BONDS	RESONANCE INTEGRAL	BOND ORDER
1	2	1	1.00	0.573
1	6	2	1.00	0.624
1	7	1	1.00	0.380
2	3	2	1.00	0.625
3	4	1	1.00	0.674
4	5	2	1.00	0.651
5	6	1	1.00	0.673
7	8	2	1.00	0.615
7	12	1	1.00	0.615
8	9	1	1.00	0.678
9	10	2	1.00	0.659
10	11	1	1.00	0.659
11	12	2	1.00	0.678
2	13	1	0.90	0.377
13	14	1	0.90	0.385
14	15	2	2.00	0.857
14	16	1	0.70	0.176
16	17	3	2.00	0.982

2-Acetamidobiphenyl

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Bond Orders for 2-amino and 2-acetamidobiphenyl